IDENTIFICATION AND CHARACTERIZATION OF COLD-REGULATED GENES IN COLD-HARDY CITRUS RELATIVE *Poncirus trifoliata* (L.) Raf

By

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To my husband Bayram

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Citrus is a cold-sensitive plant and most commercially important varieties of citrus are susceptible to freezes. On the other hand, *Poncirus trifoliata* is an interfertile *Citrus* relative that can tolerate temperatures as low as -26°C when cold-acclimated. To identify genes involved in cold tolerance in *P. trifoliata*, cDNA libraries were constructed from 2 day cold acclimated and nonacclimated *Poncirus* seedlings using a subtractive hybridization method. A total of 192 randomly picked clones, 136 from the cold induced library and 56 from the cold repressed library, were sequenced. Most of these clones showed sequence homology to previously characterized cold-induced or environmental stress-regulated genes from other plants that were deposited in GenBank.

Expression of these cDNAs was analyzed by reverse northern blot hybridization with cold-acclimated and nonacclimated probes. Analysis of expression data showed that expression of 96 cDNAs was increased 2- to 49-fold during cold acclimation. On the other hand, expression of only a few genes was repressed in response to cold. Three partial cDNAs (PI-B05, PI-C10, and PI1-C02) showing homology to previously

characterized transcription factors were selected for further characterization. The full-length cDNA sequences of these genes were obtained by 5' and 3' rapid amplification of cDNA ends (RACE). Sequence analysis revealed that PI-B05 contains the AP2 domain, PI-C10 contains the AP2 and B3 DNA binding domains and PII-C02 has a RING zinc finger domain conserved in transcription factors involved in environmental stress-induced gene expression. Expression patterns of these genes in cold-tolerant *P. trifoliata* and cold-sensitive pummelo (*Citrus grandis*) in response to cold and drought were analyzed by northern blot hybridization at different time points. Expression analysis showed that PI-B05 and PI C10 were induced only in response to cold in *Poncirus*. Expression of PII-C02 was induced by both cold and drought in *Poncirus* and pummelo.

In addition, sub-cellular localization of two previously identified cold-regulated genes, COR11 and COR19, was studied in onion epidermal cells using COR-GUS fusion constructs. Transient expression assay followed by 4',6-Diamidino-2-phenylindole (DAPI) staining showed that these proteins are localized in the nucleus and cytoplasm but accumulated predominantly in the nucleus. Deletion of the N-terminal half of COR11 demonstrated that C-terminal half conserved in both genes was sufficient for nuclear localization.

CHAPTER I
INTRODUCTION

Citrus originated in tropical regions of Southeast Asia, and have been cultivated for about 4 000 years. With annual production of more than 100 million metric tons, citrus has become one of the world's most important fruit crops. It is distributed and commercially grown widely in tropical and subtropical regions of the world. Citrus production is mostly limited by low temperatures outside of this region. Since citrus is a cold-sensitive plant, low temperatures and freezes result in significant damage and economic losses in subtropical citrus growing regions, including Florida. Although mild freezes occur relatively frequently in citrus growing regions, a number of severe destructive freezes have affected citrus production in the last century. Beside the economic losses due to destruction of citrus trees and fruits, these freezes have forced relocation of citrus production to warmer regions.

Environmental conditions often are limiting factors for plant growth and development. To cope with changes in their environment, plants have developed different mechanisms for adapting to new environments. Since agricultural production of commercially important crops is adversely affected by unfavorable environmental conditions, understanding of plants' response to environmental stresses has been an important research area. The study of environmental stress and adaptation not only provides an understanding of the environmental stress mechanisms of plants, but also allows the development of strategies for the improvement of stress tolerance in agricultural crops.

Temperature is one of the variable environmental conditions showing daily and seasonal changes. Therefore exposure to low temperature is one of the most common environmental stresses on plants Low temperatures can have a negative affect on plant growth and development. Thus, some plants have developed adaptive mechanisms, such as cold acclimation, to survive in low temperature conditions Cold acclimation is a process by which exposure to low non-freezing temperatures increases a plant's tolerance to subsequent freezing temperatures During this process, a series of measurable physiological and biochemical changes is induced by the expression of specific genes Gene-expression analysis of cold-acclimated plants showed that several hundred genes are induced during low-temperature exposure of plants including the model plant *Arabidopsis thaliana* L and economically important agricultural crops. Isolation and characterization of genes induced during cold acclimation revealed that they were involved in a variety of cellular functions Identifying these genes gives a better understanding of the mechanisms of cold tolerance, and allows the development of more cold-tolerant plants using genetic engineering

Since it is a cold-sensitive and economically important crop the development of a cold-tolerant citrus plant is an important objective for citrus industries in subtropical regions affected by freezing. In the last century, breeders discovered the existence of major cold hardiness in the cross-compatible trifoliate orange, *Poncirus trifoliata* Since then, a number of crosses between *Poncirus* and *Citrus* have been made to integrate the cold hardiness trait into commercial citrus varieties. However, even though breeding programs generated a few hybrid cultivars for rootstocks, the development of cold-

tolerant citrus scion cultivars with acceptable horticultural characteristics has yet to be successful to date

Recent developments in molecular biology have enabled the identification of key genes involved in cold acclimation and cold tolerance in plants. It has been shown in a number of different plants that cold tolerance can be improved by manipulating the expression of these genes in transgenic plants. This approach has the potential to improve cold tolerance in citrus as well. Efficient genetic transformation methods are in place for citrus. However, there is insufficient information about regulation of cold tolerance in *Poncirus* and also a lack of genes that can be used for improving cold tolerance by genetic engineering in *Citrus*. Therefore, studying gene expression during cold acclimation of *Poncirus* is needed to identify genes involved in cold tolerance and to develop cold-hardy citrus cultivars.

The overall objective of this study was to identify and characterize genes involved in cold tolerance in *Poncirus*. The specific objectives were (1) to construct subtractive cDNA libraries for identification of cold-regulated genes from cold-acclimated *Poncirus trifoliata*, (2) to analyze expression of cold-regulated genes from the cDNA libraries; (3) to isolate and clone cold induced genes from cold-acclimated *Poncirus*, (4) to study expression of selected cold-induced genes under cold and drought stresses, and (5) to demonstrate nuclear localization of two previously identified cold-induced genes (COR11 and COR19).

CHAPTER 2
LITERATURE REVIEW

Environmental Stresses

Plant species are distributed all over the world and are grown in different climates and environmental conditions. Like other organisms, plants are exposed to abiotic and biotic stresses in their environment. Thus, plant growth and development as well as crop production are highly influenced or even limited by environmental conditions such as soil and nutrition, light, temperature, and water. To survive extreme environmental conditions, plants evolved to adapt to their environment and develop mechanisms to cope with the stresses induced by these conditions. Photoperiodism, seed and bud dormancy, and low-temperature response are some examples of adaptive mechanisms that plants develop through their growth period. Many dicots and grasses promote or delay flowering in response to day length by using photoperiodism. Seeds delay developmental processes until the conditions required for germination are met using a mechanism called seed dormancy. Similarly, many temperate-zone trees stop bud growth in response to low temperatures (Taiz and Zeiger 1991), which is called endo-dormancy.

Temperature Stress

Temperature is one of the most common environmental conditions causing abiotic stress on plants. It has a significant role in the plant life cycle and is important for both developmental and physiological aspects of plants. Therefore, exposure of plants to high, low and freezing temperatures imposes significant stress to them and adversely affects their growth and development. Although high-temperature-induced stress is as important

as low-temperature-induced stress low temperature-induced stress is the focus of this review When plants are exposed to low temperatures, it causes two types of injury chilling and freezing (Stushnoff et al 1984).

Chilling Injury

Plant exposure to low but non-freezing temperatures is called chilling. Depending on the evolutionary origin of plants, chilling injury may occur at temperatures between 0 and 15°C While tropical and subtropical plants show chilling injury at 8°C and 12°C, respectively, most temperate plants show chilling injury at temperatures between 0°C and 4°C (Nishida and Murata 1996 Fowler and Limin 2002) Severity of injury during chilling is species-dependent, and plants can be divided into three different classes according to their chilling sensitivity The first group is extreme chilling sensitive plants generally tropical originating plants, including *Ephedra vulgaris* (Richt.), *Gossypium hirsutum* L., and *Vigna radiata* L. In this group chilling injury is rapid and irreversible A second group showing delayed response to chilling is called chilling-sensitive plants, such as *Cucumis sativus* L , *Glycine max* (L) Merr , *Lycopersicon esculentum* L., and *Nicotiana tabacum* L The third group is chilling-resistant plants including *Arabidopsis thaliana* L., *Brassica oleracea* L *Hordeum vulgare* L., and *Triticum aestivum* L. This group shows no chilling injury unless plants are exposed to another stress factor (Kratsch and Wise 2000).

Symptoms of chilling injuries are generally similar across species and it can cause many physiological disruptions to plants including swelling and disorganization of both chloroplasts and mitochondria, reduced size and number of starch granules, dilation of thylakoids and unstacking of grana, lipid droplet accumulation in choloroplasts, and condensation of chromatin in the nucleus (Kratsch and Wise 2000). Severity of chilling

injury depends upon different factors including light, duration of chilling, relative humidity acclimation, and certain stages of plants (Nishida and Murata 1996, Kratsch and Wise 2000). When *Selaginella* spp. were evaluated, no chilling injury was observed in the darkness. However, symptoms of chilling injury such as bleaching of chlorophyll, accumulation of lipid droplets, and degeneration of thylakoids was observed during chilling in the light (Jagels 1970). Although short exposure to chilling temperatures may not induce any injury to certain plants, longer exposure to the same chilling temperature can cause irreversible injuries (Kratsch and Wise 2000). Similarly, it was observed in both cotton and bean that high relative humidity acts as a protective factor for chloroplasts and reduces the risk of injury during chilling (Wise et al. 1983). When different developmental stages were evaluated, it was found that seedlings were more susceptible than mature plants, and the pollen developmental stage was the most sensitive to chilling temperatures (Nishida and Murata 1996).

When injury occurs during chilling, cells can die in two different ways. The first one is called necrotic cell death, which involves swelling of the cell and then lysis resulting in leakage of cellular contents. The other proposed mechanism is programmed cell death (PCD). This involves generation of reactive oxygen species that in turn trigger an increase in free Ca^{2+} ions that act as second messenger and stimulate a cascade of proteolytic enzymes involved in the cleavage of key proteins that finally results in the systematic death of the cell (Kratsch and Wise 2000).

Freezing Injury

The second type of injury caused by low temperatures is freezing injury, which occurs when the temperature drops below the freezing point of water. Exposure to low freezing temperatures may result in intracellular or extracellular freezing. Intracellular

freezing occurs when ice crystals form within the cells either by internal nucleation in the cell or by penetration of an external ice crystal into the cell. This type of freezing damages the protoplasmic structure, and growing ice crystals kill the cell. When ice forms outside of the cell, the freezing is called extracellular. In this type of freezing, water is withdrawn from the protoplast and the protoplasm becomes dehydrated. The capacity of extracellular spaces for growing ice crystals and the ability of the protoplast to withstand dehydration are key determinants of the freezing resistance in certain species. Even in this case, cooling must be slow; otherwise sudden exposure to low temperatures can cause intracellular freezing and cell death (Guy 1990, Taiz and Zeiger 1991, Fowler and Limin 2002).

Plant Responses and Adaptation to Low Temperatures

Since plants are unable to move or change their environment, they must adapt to changes in their environment. Plants have developed and used several strategies to tolerate temperature stress induced by low and freezing temperatures. Supercooling and cold acclimation are the two most common low-temperature-tolerance mechanisms by which plants protect themselves from freezing temperatures.

Supercooling

Aqueous solutions may remain in the liquid state when cooled below the freezing point which is known as supercooling. Solutions may supercool to different degrees before they spontaneously freeze. The temperature at which spontaneous nucleation occurs is termed the supercooling point or temperature of crystallization (Zachariassen and Kristiansen 2000). In the absence of any ice nucleation sites, water will remain in the liquid phase down to -38.1°C. In the presence of ice nucleation sites, ice crystals are formed and can grow up to 0°C. In some plants, water in the cell is supercooled and

maintained above the supercooling point to avoid freezing. Presence of solutes in the cell will further lower the supercooling point a few degrees, allowing plants to survive temperatures near -40 to -41 °C

Using supercooling, some woody plants can withstand temperatures of approximately -40°C by protecting critically important tissues including dormant buds and xylem ray parenchyma. As temperatures drop in the fall these plant tissues are hardened by exposure to temperatures below 0°C for several days. Although these processes are not fully understood, some modifications were observed in membrane properties. Lack of ice-nucleating sites prevents formation of ice in the protoplast of some tissues. Thus, some cells avoid freezing and show deep supercooling many degrees below the freezing point (at about -38 °C) (Fitter and Hay 2002)

Cold Acclimation

It has been shown in many plants that exposure to low non-freezing temperatures below 10°C enhances tolerance to subsequent freezing temperatures This process is called cold acclimation, and is used by many plants to cope with stress induced by low temperatures. Plants have different responses to low temperatures and maximum freezing tolerance in cold-acclimated plants is not constitutive, but is induced in response to low non-freezing temperatures (Thomashow 1998) As temperatures drop in early fall, the freezing tolerance of plants increases gradually This accelerates in late fall resulting in the maximum hardiness of plants When the temperatures increase, the cold acclimation process can be reversed, which is called deacclimation While cold acclimation takes two weeks to several months depending on the species deacclimation takes approximately a week or shorter Even though cold acclimation is a long process that takes weeks to

months molecular changes that could lead to freezing tolerance of plants can be detected at very early stages of cold acclimation (Guy 1990)

During cold acclimation, a series of physiological and biochemical changes take place in the plants which results from induction of specific gene expression (Guy 1990) These changes include the accumulation of compatible solutes such as soluble sugars, betaine, and proline alterations of membrane lipid compositions; increases in abscisic acid (ABA) concentration and antioxidant activity (Browse and Xin 2001, Smallwood and Bowles 2002).

Accumulation of Compatible Solutes

Plants accumulate low molecular weight organic solutes such as sugars, proline and glycine betaine in response to low temperature and other stress conditions that cause depletion of cellular water including drought and high salinity During cellular dehydration, these compounds increase osmotic pressure and prevent loss of water from cells. These organic solutes are called compatible solutes and have a hypothesized function as cryoprotectants by preventing protein denaturation. They maintain membrane integrity by interacting with polar head groups of phospholipids or forming hydrophobic interactions with the membrane They are also osmolytes that lower the freezing point of cytosol (Guy 1990, Hare et al 1998 Smallwood and Bowles 2002)

Accumulation of sugars, primarily sucrose and the raffinose families of oligosaccharides (RFO) such as raffinose and stachyose were observed in many plants in response to environmental stresses including exposure to low temperatures (Hinesley et al. 1992; Holaday et al 1992, Castonguay and Nadeau 1998) These increases are correlated with the increased activity of enzymes involved in the synthesis of these sugars (Guy 1990) Sucrose-phosphate synthase (SPS) and galactinol synthase (GS) are the key

enzymes in the sucrose and RFO biosynthetic pathways, respectively. A correlation between increased activities of SPS and GS and the levels of sucrose and RFO, respectively was reported in alfalfa (Castonguay and Nadeau 1998). In addition, an increase in the endogeneous levels of glucose, fructose, raffinose, and stachyose was observed at the onset of cold acclimation, in August and their levels reached a maximum in December and January in grapes (Hamman et al. 1996).

Sugars might have a role in osmoregulation by increasing intracellular osmotic potential (Crow et al. 1993). This process lowers the freezing temperature and prevents drying of the cells by retaining more liquid water inside the cell, which provides a longer period of metabolic activity during extracellular freezing (Levitt 1980). Sugars also have a role in cryoprotection of cell membranes and proteins (Guy 1990). They act as cryoprotectants by protecting cell membranes from high concentrations of electrolytes (Crowe et al. 1990).

Glycinebetaine is another compatible solute that accumulates in response to many stresses including low temperature (Allard et al. 1998), salinity (Grieve and Maas 1984), and drought (Ladyman et al. 1983). Based on *in vitro* studies, glycinebetaine appears to stabilize the structures of enzymes and complex proteins and maintain the integrity of membranes under stress conditions (Sakamoto and Murata 2001, Smallwood and Bowles 2002). It is synthesized from choline by a two step oxidation reaction. During the first oxidation reaction of choline, betaine aldehyde is obtained by the activity of choline monooxygenase enzyme. Then, glycinebetaine is synthesized by the activity of betaine aldehyde dehydrogenase enzyme during the second oxidation step of choline (Russell et al. 1998, Iba 2002). The genes encoding both enzymes have been cloned from many

plants including barley (Ishitani et al., 1995), rice (Nakamura et al 1997), and spinach (Rathinasabapathi et al 1997)

Since exogenous application of glycinebetaine enhances stress tolerance (Mäkelä et al 1998) choline monooxygenase and betaine aldehyde dehydrogenase have been introduced into plants separately to obtain glycinebetaine. These studies were not successful because sufficient amounts of choline substrate and betaine aldehyde were unavailable in the cell (Nuccio et al 1998, Iba 2002). When a heterologous betaine aldehyde dehydrogenase gene was introduced into rice along with exogenous betaine aldehyde transgenic rice plants accumulated glycinebetaine and showed resistance against salt and low temperatures (Kishitani et al 2000) Another way to obtain glycinebetaine is via oxidation of choline by choline oxidase (COD) in a one-enzyme catalyzed reaction in the soil bacteria, *Arthrobacter globiformis* and *Arthrobacter pascens* The CodA and cox genes, which encode COD of *A globiformis* and *A pascens* respectively, have been introduced into many plants including *Arabidopsis* (Hayashi and Murata 1998) rice (Sakamoto et al. 1998), tobacco (Huang et al 2000), and *Brassica napus* (Huang et al 2000) These transgenic plants showed improved tolerance to several abiotic stresses including low-temperature stress (Sakamoto and Murata 2001). In the transgenic plants, the phase transition from the liquid crystalline to the gel state was shifted (Sakamoto and Murata 2001 Smallwood and Bowles 2002).

Plants accumulate proline in response to different environmental stresses (Hare et al 1999) including low temperature (Barka and Audran 1997). There are several roles of proline in plant stress tolerance including osmoregulation (Yoshiba et al 1997) and induction of osmotically regulated genes (Iyer and Caplan 1998), stabilization of proteins

and membranes (Rudolph et al 1986) as well as protection against reactive oxygen species (ROS) (Smirnoff and Cumbes 1989) Proline accumulation was observed after exogenous application of ABA to many plants including barley (Pesci 1987) and maize (Xin and Li 1993) Based on studies with ABA mutants, proline accumulation can be observed through both ABA-dependent and ABA independent regulation mechanisms (Nambara et al 1998) A correlation between the accumulation of proline and improved cold tolerance has been observed in many plants including wheat (Dörffling et al 1997), grapevine (Barka and Audran 1997), and *Arabidopsis* (Xin and Browse 1998) In addition, some mutants with constitutively high levels of proline showing enhanced freezing tolerance have been isolated, for example the *Arabidopsis* eskimo1 mutant (*esk1*) After cold acclimation *esk1* plants maintained their high level of proline, which is 30-fold higher than cold-acclimated wild-type plants (Xin and Browse 1998)

Alterations of Lipid Composition and Membrane Structure

Studies of freezing and chilling injuries have indicated that membranes are the primary target Exposure to low temperatures causes damage to the plasma membrane due to cellular dehydration Several types of membrane damage can result from freezing injury to plants, including expansion induced lysis, lamellar to-hexagonal II (H_{II}) phase transitions, and fracture jump lesions (Thomashow 1998) In expansion-induced lysis, freezing and thawing induce expansion and eventually lysis of protoplasts During freezing of the medium water diffuses out of the protoplast, reducing the tension in the cytoplasm, which results in endocytotic vesiculation of the plasma membrane Formation of vesicles does not cause injury, but it significantly reduces the surface area of the plasma membrane. When the medium thaws, water diffuses into protoplasts increasing the cytoplasmic volume and resulting in re-expansion of the plasma membrane. The

vesicles are not readily re-incorporated, leading to lysis of the protoplasts (Dowgert and Steponkus 1984). Under non-freezing conditions, polar head groups of phospholipids are oriented in the aqueous core of the lipid bilayer of the membrane. During freezing, low-temperature-induced dehydration causes a phase transition from lamellar to the H_{II} phase in which three-dimensional discontinuous structures with long tubes of lipids are formed. The formation of the H_{II} phase disrupts the membrane structure by changing continuity and semipermeability of the plasma membrane, leading to solute leakage and introduction of ice nucleation sites into the cells (Gordon-Kamm and Steponkus 1984). In some plants, instead of an H_{II} phase transition, fracture jump lesions were observed in localized regions in which the fracture plane in the plasma membrane has "jumped" to lamellae of the endomembranes that are in close apposition to the plasma membrane. (Webb and Steponkus 1993).

Chilling also induces structural changes in cell membranes. It has been reported that membrane permeability was increased in response to chilling. This causes ion leakage and altered ion balance in chilling sensitive tissues. During chilling, phase changes (liquid to solid) occur in membrane lipids, which results in deactivation of membrane-bound enzymes, leading to slower respiration, inhibition of photosynthesis and protein synthesis, and increased degradation of proteins (Taiz and Zeiger 1991; Nishida and Murata 1996; Fitter and Hay 2002). It has been reported that cold acclimation prevents the membrane damage caused by freezing and chilling injuries, including expansion-induced lysis and formation of H_{II} phase transition in rye (Steponkus et al. 1990). Stabilization of membranes during cold acclimation can be attributed to several factors, including changes in lipid composition, accumulation of sugars, and

dehydrins. It was suggested that dehydrins stabilize the membranes through hydrophobic interaction with phospholipids (Close 1996, 1997) and may protect integrity of membranes by preventing lamellar-to-hexagonal phase transitions (Thomashow 1999). It has been shown that in plants and other organisms, the level of unsaturated fatty acids increases at low temperature. Increases in unsaturation of membrane lipids compensates for the reduced membrane fluidity and enzyme activity caused by low temperature. A correlation between chilling sensitivity of plants and unsaturation of membrane lipids was observed and studies have indicated that plants containing higher percentages of unsaturated fatty acids generally showed more chilling resistance (Nishida and Murata 1996). Therefore, enzymes involved in unsaturation of membranes, such as acyltransferases and desaturases from plants and microorganisms, have been used for improving cold tolerance in plants (Nishida and Murata 1996, Iba 2002).

Changes in ABA Level

An increase in the level of ABA in response to low temperature was first observed in cold-acclimating *Solanum commersonii*. This increase was transient and was not observed in non-acclimating plants. In addition, exogenous application of ABA increased the freezing tolerance of *S. commersonii* at warm temperatures (Chen et al. 1983). Later, it was shown that ABA levels increased in response to low temperature in other plants (Lalk and Dörffling 1985; Luo et al. 1992; Lång et al. 1994), and exogeneous application of ABA increased the freezing tolerance of many plants (Orr et al. 1986, Ishikawa et al. 1990). Exogeneous application of ABA also increased the expression of COR genes, including COR78, COR47, COR15a, and COR6.6 (Hajela et al. 1990; Kurkela and Franck 1990; Gilmour et al. 1992; Nordin et al. 1993; Gilmour et al. 1992, Yamaguchi-Shinozaki and Shinozaki 1993). It was shown that expression of some cold

regulated genes, such as *Arabidopsis* RAB18 and rd29A, was dependent on ABA and that these genes carry putative ABA-responsive elements (ABREs) in their promoters (Lång et al 1992, Nordin et al 1993 Wellin et al 1994, Yamaguchi-Shinozaki and Shinozaki 1994). Gene expression through ABREs is regulated by bZIP transcription factors and several cold-responsive bZIP proteins were identified in plants, including *Arabidopsis* (Lu et al. 1996 Choi et al 2000) rice (Aguan et al 1993), and maize (Kusano et al 1995) Studies on the *abi1* mutant in *Arabidopsis* showed that the ABA-induced accumulation of these COR genes was eliminated but cold induced accumulation of these transcripts was not affected in this mutant. Consequently, it was concluded that cold-regulated expression of these genes was controlled by an ABA-independent pathway (Gilmour et al. 1991, Nordin et al 1991)

Changes in Antioxidant Activity

Active oxygen species (AOS) such as superoxide anions (O^-2) and hydrogen peroxide ($H2O2^-$) are produced by the reduction of molecular oxygen (O2) and the hydroxyl radicals (OH•) which are produced in cells under stress conditions (Iba, 2002). Levels of AOS increase at low temperature, causing oxidative stress in the cell Oxidative stress damages cells in different ways, including lipid peroxidation, membrane deterioration, protein and nucleic acids degradation, and chlorophyll quenching (Scandalios 1990 Bestwick et al 1998 Pastori et al 2000) Plants and other organisms produce a variety of antioxidants to control AOS levels and to prevent oxidative stress in their cells Antioxidants can be divided into three groups. The first group contains lipid soluble and membrane-associated tocopherols The second group includes water soluble reductants, such as ascorbic acid and glutathione The last group is includes the antioxidant enzymes- superoxide dismutase (SOD) catalase (CAT) ascorbate peroxidase

(APX) monodehydroascorbate reductase (MDAR) dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Foyer 1993 Iba 2002).

It has been observed that the levels of antioxidants in many plants increase at low temperatures during cold acclimation to prevent or reduce the potential damage of increased levels of AOS It has been shown that the antioxidant enzymes SOD APX, CAT, MDAR, DHAR, and GS are involved in AOS-detoxification systems in plants (Iba 2002) SOD catalyzes the dismutation of two superoxide radicals into oxygen and hydrogen peroxide. The APX and CAT detoxify the hydrogen peroxide by converting it to water In addition, APX oxidizes ascorbic acid and MDAR, DHAR, and GS are involved in ascorbic acid recovery Although CAT is localized only in the microbodies in almost all plant species, the other enzymes are found as multiple isozymes at different locations in the cell According to the metal cofactor involved, SOD can be divided into three groups Mn SOD, Fe-SOD, and Cu,Zn-SOD which are localized in the mitochondria, chloroplast, and chloroplast and cytosol, respectively (Holberg and Billow 1998 Iba 2002). It was shown that APX and GR levels increase when H2O2 accumulates in the cell under low-temperature conditions in Arabidopsis (O Kane et al 1996) and Fe-SOD and CuZn-SOD levels increase when tobacco plants are chilled (Tsang et al 1991). Since these enzyme levels increase in stress conditions they were overexpressed in transgenic plants to study their roles in stress tolerance When chloroplast CuZn-SOD was overexpressed, resistance to intense light and low temperature was improved in transgenic tobacco plants (Gupta et al 1993a; Gupta et al 1993b) Overexpression of Mn SOD and Fe-SOD in transgenic alfalfa also resulted in improved resistance to low temperature (McKersie et al 1999, McKersie et al 2000)

However, transgenic tomato plants overexpressing Escherichia coli GR showed no improvement in chilling tolerance (Brüggemann et al. 1999).

Changes in Gene Expression

Physiological and biochemical changes leading to freezing tolerance in plants have been associated with changes in gene expression during cold acclimation. Although this has been postulated and demonstrated to a point for many years, changes in gene expression during cold acclimation has been only recently studied extensively in many plants. It was first shown that there is a correlation between accumulation of soluble proteins in cortical bark cells of black locust trees and freezing tolerance (Sminowitch and Briggs 1949). Weiser, (1970) proposed that maximum freezing tolerance of temperate woody perennials is associated with changes in gene expression and synthesis of new proteins during cold acclimation. Guy et al. (1985) demonstrated that expression of a number of genes was altered in spinach during cold acclimation. Since then, many cold-regulated genes have been isolated in many plants including *Arabidopsis* (Gilmour et al. 1998, Lin et al. 1998), *Brassica napus* (Sáez Vásquez et al. 1995), *Hordeum vulgare* (Dunn et al. 1996), *Medicago sativa* (Castonguay et al. 1994), *Spinach oleracea* (Guy and Li 1998) and *Poncirus trifoliata* (Cai et al. 1995). Isolation and characterization of these genes are important not only for understanding the low temperature response and the mechanism of cold tolerance in plants, but also for improving cold tolerance in agricultural crops resulting in increased plant productivity and expanded areas of agricultural production.

Cold acclimation has an effect on the expression of many genes; these genes are altogether called cold-regulated genes. The cold-regulated genes have been referred to with different terms in the literature based on the specific response of the gene under

certain conditions or by the preference of the groups working with the genes responsive to dehydration (rd) early responsive to dehydration (erd) cold induced (kin), cold regulated (cor), and low temperature induced (lti) Most low temperature and dehydration inducible genes are in common because low temperature reduces the water potential of the extracellular compartment which causes loss of water from cell by osmosis leading to dehydration. In addition low temperature decreases turgor pressure as a result of dehydration, which results in the induction of the plant stress hormone ABA Thus, some genes can be responsive to cold, dehydration, and the exogenous application of ABA, while others are induced in response to cold and dehydration stress, but not by ABA treatment. It was suggested that cold-regulated genes could be induced by different pathways and it was shown that both ABA-dependent and ABA-independent pathways exist and are involved in cold-regulated gene expression in plants (Shinozaki et al 2000 Viswanathan and Zhu 2002)

The genes induced in response to low temperatures during cold acclimation can be divided into two groups those directly involved in freezing protection and those that regulate gene expression. Genes in the first group are COR genes involved in freezing tolerance, and include COR6 6, COI5a, CO47, and COR78 isolated from *Arabidopsis* (Hajela et al 1990) Based on DNA sequence analysis, it was shown that COR6 6 COR15a, and COR78 encode novel hydrophilic polypeptides and COR47 encodes a group II late embryogenesis abundant (LEA) protein (Thomashow 1998) that thought to function in dehydration tolerance (Close 1997) In general, COR genes show biochemical similarities with cryoprotective proteins (Artus et al 1996) which are plant leaf proteins that were reported for the first time in cabbage and spinach leaves and

function in protecting isolated thylokoid membranes from freeze-thaw damage (Volger and Heber 1975). These COR proteins are hydrophilic and synthesized in response to low temperature and remain soluble upon boiling (Artus et al. 1996). Homologs of these genes and many other cold regulated genes have been identified and isolated from different plants. These genes encode for novel hydrophilic proteins including BN115 from *Brassica napus* (Weretilnyk et al. 1993), CR1 from *Hordeum vulgare* (Cattivelli and Bartels 1990), CAS15a from *Medicago sativa* (Monroy et al. 1993), CAP160 from *Spinach oleracea* (Kaye et al. 1998) and hydrophobic polypeptides including RC12A from *Arabidopsis* (Capel et al. 1997), tacr7 from *Triticum aestivum* (Gana et al. 1997) or homologs of LEA proteins including ERD14 from *Arabidopsis* (Kiyosue et al. 1994), cas18 from *Medicago sativa* (Wolfraim et al. 1993), CAP85 from *Spinach oleracea* (Neven et al. 1993), and COR15 and COR19 from *Poncirus trifoliata* (Cai et al. 1995).

To test if COR15a had a role in freezing tolerance and whether it had the same function as cabbage and spinach proteins, transgenic plants constitutively expressing COR15a were produced. It was shown that chloroplasts of transgenic plants were 1°C to 2°C more freezing tolerant than the chloroplasts of wild type plants (Artus et al. 1996). Later experiments established that COR15a helps stabilize membranes during freezing by decreasing freeze induced lamellar to-hexagonal II phase transition (Steponkus et al. 1998). Even though constitutive expression of COR15a increased the freezing tolerance of transgene plants, this increase was small which was expected considering the quantitative nature of cold tolerance.

More comprehensive studies involving microarray analyses of thousands of genes revealed that up to several hundred genes were regulated during cold acclimation. These

genes are involved in a variety of cellular functions including metabolism, transcription protein fate, transport facilitation, biogenesis, communications and signal transduction, cell rescue and defense, cell death and aging (Seki et al 2001 and 2002, Fowler and Thomashow 2002)

Analysis of the regulatory sequences of COR genes revealed that promoters of COR15a and COR78 contained a TGGCCGAC regulatory sequence element which was activated in response to low temperature and dehydration stress (Horvath et al 1993) This element was termed the C-repeat (CRT) (Baker et al 1994) At the same time, Yamaguchi-Shinozaki and Shinozaki (1994) found that the promoter of RD29A (COR78/LTI78) contained a regulatory TACCGACAT *cis*-element that has a role in both dehydration and cold responsive gene expression and they called it a dehydration responsive element (DRE) Both CRT and DRE elements have the same five base pair (bp) core sequence, CCGAC and multiple copies are found in the promoters of many cold and dehydration-regulated genes

Characterization of regulatory sequences of the cold and dehydration response genes provided researchers with necessary information for the identification of the regulatory protein binding to these sequences. A regulatory protein binding to the CRT/DRE element was isolated using a yeast one hybrid screen and was designated the CRT/DRE Binding Factor 1 (CBF1) (Stockinger et al 1997) It has a molecular mass of 24 kDa, a putative bipartite nuclear localization signal an AP2 DNA-binding domain which contains a 60 amino acids (aa) motif present in a number of plant proteins including *Arabidopsis* APETAL2, AINTEGUMENTA, and TINY, as well as the tobacco ethylene element response binding proteins (EREBP's) and an acidic region that

functions as an activation domain (Stockinger et al. 1997). Two additional members of CBF proteins were isolated by screening an *Arabidopsis* cDNA library prepared from cold-acclimated plants and were designated CBF2 and CBF3. Based on the aa sequences of CBF proteins, they were 88% identical and 91% similar to each other (Gilmour et al. 1998).

Independently, Liu et al (1998) isolated two proteins binding to DRE elements, also using a yeast one-hybrid screening technique, and called them DRE-binding proteins (DREB1A and DREB2A), which were induced in response to low temperature and dehydration and high salt stresses, respectively. Like CBF proteins, both DRE binding proteins contain an EREBP/AP2 domain. Except for the similarities found in the DNA binding domain, there is no significant sequence homology between the two DREB proteins. Using DREB1A sequences, two more DRE binding proteins designated DREB1B and DREB1C were isolated (Liu et al. 1998). The DRE binding proteins DREB1B, DREB1C and DREB1A are identical to the CRT binding proteins CBF1, CBF2 and CBF3, respectively, and regulate expression of genes containing the CRT/DRE sequence element in their promoters (Stockinger et al. 1997, Liu et al. 1998, Gilmore et al. 1998, Shinwari et al. 1998) in response to both low temperatures and dehydration through an ABA independent pathway (Yamaguchi-Shinozaki and Shinozaki 1994). These three genes form the CBF/DREB protein family. They were mapped in a direct repeat on chromosome 4 in the order CBF1/DREB1B, CBF3/DREB1A, and CBF2/DREB1C (Gilmore et al. 1998, Shinwari et al. 1998). The activities of all CBF/DREB proteins change depending on the COR promoter sequences. This result indicates that CBF binding to the CRT/DRE sequence depends not only on the CCGAC

core CRT sequence, but also on nucleotides outside of this core sequence (Gilmore et al. 1998)

Since CBF/DREB proteins are involved in regulation of cold and dehydration responsive genes these proteins were expressed in transgenic plants for improving cold tolerance Overexpression of CBF/DREB proteins under the control of the constitutive CaMV35S promoter increased freezing tolerance in transgenic Arabidopsis plants (Jaglo-Ottesen et al 1998; Liu et al 1998 Kasuga et al 1999; Gilmour et al 2000) When CBF1 was expressed in transgenic Arabidopsis plants, it induced the expression of COR genes containing the CRT/DRE element, including COR6 6 COR15a, COR47, and COR78, without a low temperature stimulus, and increased the freezing tolerance of non-acclimated Arabidopsis plants. (Jaglo-Ottesen et al 1998) Freezing and dehydration tolerance were also observed in transgenic Arabidopsis plants overexpressing DREB1A and DREB2A, which was correlated with the level of expression of the stress inducible genes under unstressed conditions In these plants, the expression level of RD29A was induced more than in the wild type plant under unstressed or stress conditions including, low temperature dehydration, high salt and ABA treatment However, transgenic plants expressing DREB1A and DREB2A showed severe and mild growth retardation, respectively (Liu et al 1998)

Arabidopsis plants overexpressing CBF3 showed that transcript levels of CBF3 were almost equal in nonacclimated and cold-treated transgenic plants, but were higher than in nonacclimated or cold treated control plants. In addition, expressions of COR genes COR15a and COR6 6 were similar in nonacclimated and cold-treated transgenic plants as well as in cold-acclimated control plants. These transgenic plants also have a

dwarf phenotype with shorter leaves compared to the control plants and require more time for flowering. Increased freezing tolerance was observed in both nonacclimated and cold-acclimated CBF3-expressing plants compared to non-acclimated or cold acclimated control plants. They also accumulated proline Δ1-pyrroline 5-carboxylate synthase (P5CS), which is a key enzyme in determining the proline level in plants and soluble sugars including sucrose, raffinose, glucose and fructose. These results indicated that over-expressing CBF3 leads to multiple biochemical changes that generally occur in plants during cold acclimation. Thus, CBF3 appears to be a key regulatory gene that functions in activating multiple mechanisms resulting in increasing the freezing tolerance of plants (Gilmour et al. 2000).

These overexpression studies demonstrated that freezing tolerance, which is a quantitative trait involving many genes, can be manipulated and improved by the expression of single regulatory genes. However, the expression of these regulatory genes under strong constitutive promoters are physiologically costly to the plant, resulting in undesirable growth phenotypes such as those observed in transgene plants overexpressing CBF3/DREB1A. To attempt to reduce adverse affects of overexpression of CBF/DREB proteins in transgenic plants, Kasuga et al. (1999) expressed DREB1A under the stress inducible promoter of the rd29A gene in Arabidopsis. These transgene plants showed a greater improvement in drought, salt and cold tolerance, and less or a minimal level of growth retardation compared to transgene plants overexpressing the same gene under a strong constitutive promoter (Kasuga et al. 1999).

Once the importance of CBF/DREB transcription factors in regulation of cold responsive genes was established in *Arabidopsis*, the presence of this pathway and

homologs of CBF/DREB proteins were investigated in other plants. CBF/DREB-like proteins were identified and characterized in *Brassica napus*, a close relative of *Arabidopsis* (Jaglo et al. 2001, Gao et al. 2002) and cold-acclimated wheat, rye (Jaglo et al. 2001) and barley (Choi et al. 2002), as well as tomato which does not cold acclimate (Jaglo et al. 2001). As in *Arabidopsis*, transcripts of CBF/DREB-like genes in *B. napus*, wheat, rye, and barley accumulated 15-30 min after exposuring plants to low temperature (Jaglo et al. 2001, Choi et al. 2002). After the expression of these genes, expression of Bn115, an ortholog of *Arabidopsis* COR15a, and Wes120/COR39 orthologues of *Arabidopsis* COR47 were induced in *B. napus*, wheat, and rye, respectively. In addition, transgenic *B. napus* plants that constitutively overexpressed *Arabidopsis* CBF genes showed accumulation of Bn115 and Bn28 without a low temperature stimulus and the freezing tolerance of both nonacclimated and cold-acclimated plants was increased. Based on these findings, it was concluded that the CBF/DREB cold response pathway is conserved among a number of plants including cold-sensitive and nonacclimating plants (Jaglo et al. 2001, Choi et al. 2002).

In *Arabidopsis*, transcript levels of all three CBF/DREB proteins increased within 15 min and accumulation of COR gene transcripts was observed at 2 h after transferring plants to low temperature. Based on sequence analyses, promoter regions of the three CBF genes are 30% identical in DNA sequence and they have a number of small deletions and insertions. No CRT sequence element was observed in the promoters of any of the CBF genes, indicating that these genes are not auto-regulated. This was confirmed in transgenic plants overexpressing CBF1 in which no accumulation of CBF3 transcripts was observed. Although CBF promoters do not contain a CRT sequence, they

contain some possible cold regulatory elements including multiple copies of Myc recognition, CANNTG and ACGT core G-box sequences. In addition to these cold regulatory elements, all three promoters contain an internally repetitive sequence ACAATTANNACAATTT approximately at the same position (Gilmore et al. 1998).

Since the expression of CBF/DREB genes was induced within 15 min of cold acclimation it was proposed that an unknown activator of CBF/DREB genes which is called inducer of CBF expression (ICE) should be present in the cell. It was further suggested that ICE would be in an inactive state at warm temperature, and under low temperature conditions, it would be activated and induce transcription of CBF/DREB genes by binding to CBF/DREB promoters (Gilmour et al. 1998). Recently, an inducer of CBF expression 1, (ICE1), which is an upstream transcription factor regulating the transcription of CBF genes in the cold, was identified and cloned. ICE1 encodes a MYC-like bHLH transcription factor and binds to MYB recognition sequences in the promoter of CBF3. It was found that ICE1 was a positive regulator of CBF3 and a mutation in ICE1 abolished CBF3 expression and decreased the expression of many CBF target genes, resulting in a reduction in the chilling and freezing tolerance of the mutant. Expression of CBF1 and CBF2 genes are less affected in the ICE1 mutant. Overexpression of ICE1 in wild type plants induced the expression of the CBF regulon in the cold, which resulted in increasing freezing tolerance of transgenic plants (Chinnusamy et al. 2003).

Methods Used for Identification of Cold Regulated Genes

The main goal of molecular cold acclimation studies is to identify genes expressed during cold stress. The most common way to identify genes expressed differentially is to compare expression of those genes under two different conditions. For

identification of cold regulated genes expression studies were conducted under normal and cold-acclimated conditions in many plants including *Arabidopsis* (Gilmour et al. 1992, Horvath et al. 1993), alfalfa (Monroy et al. 1993), barley (Dunn et al 1991, Hong et al 1992), *Brassica napus* (Weretilnyk et al 1993) and wheat (Chauvin et al 1993) The differences in gene expression under different conditions have been studied using a variety of methods. Northern blot hybridization (Cai et al 1995), dot blot hybridization (Mrac et al 1997) semi quantitative (Sorrell et al. 2002) and quantitative reverse transcription-polymerase chain reaction (RT-PCR) (Massonneau et al 2001) were used for expression studies of single or a few differentially expressed genes. In the last decade subtractive hybridization (Wang et al 2001), differential display (Horvath and Olson et al 1998) serial analysis of gene expression (SAGE) (Matsumura et al. 1999) and sequencing of expressed sequence tags (ESTs) (Zhang et al. 2001) have been developed to study expression levels of many genes between and among different experimental conditions at the same time More recently, macroarray and microarray analyses have been developed and used for the global analysis of the expression of thousands of genes simultaneously under different experimental conditions (Moody 2001) Although gene arrays are presently the most powerful methods for studying gene expression on a wide scale, they require the availability of sequence information and the necessary equipment is expensive for most laboratories. Sequence information is limited for most agricultural crops, including citrus, therefore, sequences must be generated using different methods such as construction and sequencing of ESTs and subtractive libraries for specific condition before using macro- or microarrays for identifying novel genes.

Subtractive Hybridization

Subtractive hybridization is a very efficient method for enrichment and isolation of differentially expressed genes It was first described in the early 1980s for construction of cDNA libraries (Sargent and Dawid 1983) and preparation of probes (Davis et al 1984) of differentially expressed genes The main goal of the subtractive hybridization is to identify differentially expressed genes by hybridizing the cDNA from one treatment (tester) with an excess amount of mRNA from another treatment (driver) During hybridization, transcripts present in both tester and driver form an mRNA/cDNA hybrid however, cDNA sequences present only in the tester do not hybridize with mRNA from the driver and stay single stranded Then, single stranded cDNAs representing differentially expressed genes are separated from double stranded nucleic acids by hydroxylapatite chromatography and cloned and used for identification of novel genes Initially the use of subtractive hybridization was limited by the requirement of large quantities of mRNA and a bias against the identification of rare transcripts (Moody 2001). To improve the recovery of differentially expressed genes some modifications such as tagging the cDNA with biotin (Welcher et al 1986), or oligo(dT)30-latex (Hara et al 1991) were introduced into the subtractive hybridization method The initial amount of mRNA required for subtractive hybridization was greatly reduced by adaptation of generic linkers to cDNA (Duguid and Dinauer 1990) and selective PCR amplification of tester cDNA between hybridization cycles (Hara et al 1991). In addition to reducing the initial amount of mRNA required for subtractive hybridization, these improvements increased the efficiency of differentially expressed genes The bias against the identification of rare transcripts was overcome by introduction of the Suppression Subtractive Hybridization (SSH) PCR technique in which differentially

expressed sequences are selectively amplified and amplification of abundant transcripts are suppressed (Diatchenko et al 1996) The SSH PCR eliminated the need for separation of single and double stranded molecules and improved the efficiency of identification of rare transcripts through normalization of differentially expressed genes by suppressing abundant and enriching the rare transcripts (Mandy 2001, Ji et al 2002) The SSH PCR method has been commercialized and developed as PCR Select cDNA Subtraction Kit by Clontech (Clontech Palo Alto, CA), which has been used widely in the identification of differentially expressed genes in different organisms (Duchaile et al 2000 Robert et al. 2000 Leypoldt et al 2001) including plants (Wang et al 2001, Matvienko et al 2001, Wang et al 2002 Takemoto et al 2003) Subtractive hybridization has been useful for gene expression studies and for the identification of differentially expressed genes on a global scale without any prior sequence information. Compared to other methods, subtractive hybridization produces fewer false positives however it requires further studies for verification of differential expression of identified genes by northern blot analysis with gene specific probes or reverse northern blot hybridization using cDNA array analysis.

Array Analysis of Gene Expression

A microarray is a grid of DNA spots on a chip, small glass slide, or nylon membrane which is used for hybridization to determine the level of gene expression. There are three general types of microarrays including oligonucleotide chips, oligonucleotide arrays and cDNA arrays The oligonucleotide chips contain short oligos directly synthesized and fixed on a glass wafer using photoactivated chemistry The oligonucleotide arrays contain presynthesized oligos spotted on glass slides or to nylon

membranes. The cDNA arrays contain PCR amplified inserts from cDNA or EST clones placed on glass slides or nylon membranes (Aharoni and Vorst 2001, Moody 2001)

Gene expression profiling is the most widely used application for microarrays thus can be used to obtain functional information for genes with unknown function. Statistical techniques such as hierarchical clustering principal component analysis (PCA) and self-organizing maps (SOM) are used for grouping genes based on their expression profiles from microarray data. Hierarchical clustering of gene expression analysis uses a bottom-up approach to join genes with similar expression profiles to form nodes, which are in turn further joined The joining process continues until all genes are combined in a single hierarchical tree based on their expression profiles. The data obtained from these analyses can give information about cellular regulatory mechanisms, and can group unknown genes with the same putative function (Aharoni and Vorst 2001).

The increasing amount of sequence information available makes microarray analysis one of the most important tools for functional genomics to close the gap between sequence information and functional identification of genes in plants. Therefore, using microarray analysis of differentially expressed plant genes in many developmental stages, different conditions, and genotypes can be classified into different groups based on their expression patterns (Kuhn 2001) In plants, microarray technology was first applied to *Arabidopsis* to study and compare gene expression in leaf tissue and roots using 48 cDNA sequences (Schena et al. 1995) Later a cDNA microarray containing 1483 *Arabidopsis* genes was used to find gene expression profiles in different organs and different developmental stages (Ruan et al 1998) Recently, microarray analysis has been widely used in *Arabidopsis* and other plants including rice, maize, petunia,

strawberry, and lima beans, (*Vicia* spp) to identify genes induced in plant defense, fruit ripening, circadian clock regulation, phytochorome A signaling, seed development, nitrate assimilation, and environmental stress (Aharoni and Vorst 2001) Microarray analysis has recently been used to identify genes involved in cold and drought response in *Arabidopsis* (Seki et al 2001, Seki et al 2002 Fowler and Thomashow 2002), barley (Hazen et al 2003), and sugarcane (Nogueira et al 2003) Since availability of sequence information is a limiting factor for microarray analysis, its application can be extended in different plants as more sequences become available

Cold Response in Citrus

Citrus is a fruit crop that grows in tropical and subtropical regions of the world. Most commercial citrus types are susceptible to low temperature, however they are able to cold acclimate to some extent Among the commercial citrus species there is variation in cold sensitivity; limes, lemons and pummelos are the most cold-sensitive types followed by grapefruits, oranges, and mandarins Pummelo is one of the most cold-sensitive types and can survive only at -4 or -5°C On the other hand mandarins such as 'Satsuma', which is the most cold-tolerant of the commercial citrus, can survive temperatures as low as -10 °C (Yelonosky 1985 Jackson and Fasulo 1994) Since most citrus species are cold- sensitive, production of citrus is mostly limited by low temperatures outside tropical and subtropical regions.

Significant economical losses from freezing have been reported in subtropical citrus growing regions including Florida in the last century These freezes have destroyed significant portions of citrus trees and fruits in certain regions and in some cases several times over Six major impact freezes between 1835 and 1989 has forced relocation of citrus production further to the south in Florida (Attaway 2000) The extent

of cold damage in citrus during/following a freeze depends on a number of different factors, including the severity of the freeze, tree/orchard location, tree dormancy, tree vigor, scion and rootstock combination, crop load, and soil conditions, as well as, freeze duration. Freezing induces injury symptoms in different parts of the plant. These symptoms include dark water-soaked areas on the leaves, leaf fall, bark splitting, and freeze cankers on tree trunks. In addition, freezes can cause fruit drop, pitting on the surface of the fruits, and extensive internal fruit injury (Jackson 1994). A number of strategies ranging from the simple burning of lighter wood, to tree wraps, to use of relatively advanced microsprinklers have been used for freeze protection in citrus. Although some of these methods provide protection from freezes, their use requires advanced freeze warnings, which require sophisticated freeze forecasts. Even though these strategies provide a certain degree of freeze protection, better protection against freezes requires development of cold tolerant citrus varieties.

A need for the development of cold-hardy citrus varieties was recognized after the freezes of 1894-95 destroyed a significant portion of the citrus in Florida. A breeding program for improving cold tolerance in commercial citrus was initiated using *Poncirus trifoliata*. This is an interfertile citrus relative that can withstand temperatures down to -26°C when cold acclimated. Since that time it has been used in breeding programs to develop cold-hardy citrus varieties. Using *Poncirus trifoliata* as a parent, several citranges including 'Rusk', 'Morton', 'Savage', and 'Troyer' were developed in the cultivar improvement programs and 'Troyer' citrange has been used as a cold tolerant rootstock (Cameron and Frost 1968; Soost and Cameron 1975; Soost and Roose 1996). More recently, progenies from open pollinated pummelo x *Poncirus trifoliata* hybrids have

been generated to produce freezing resistant citrus cultivars (Yelenosky et al 1993). However, production of commercial scion varieties with good fruit quality and cold hardiness have been largely unsuccessful to date because of biological problems associated with citrus breeding including long juvenility periods, polyembryony, heterozygosity sterility self and cross incompatibility, inbreeding depression, and the quantitative inheritance of cold tolerance

Traditional breeding methods have provided limited information about the genetics of cold tolerance and have not been successful for developing cold tolerance in citrus. To overcome the limitations of conventional breeding and improve understanding of important genetic traits in citrus, molecular markers have been integrated into breeding programs. Using molecular markers, a number of genetic linkage maps were developed in citrus and *Poncirus* hybrid populations (Durham et al 1992 Jarrell et al. 1992 Cai et al 1994, Liou et al 1996 Sahin-Cevik 1999, Sankar and Moore 2000 Weber et al 2003) The genetics of cold tolerance was studied using quantitative trait loci (QTLs) mapping in a *Citrus grandis* x *Poncirus trifoliata* F_1 pseudo-testcross population In this study, a QTL with a major effect and several more QTLs with smaller effects on cold tolerance were identified using QTL mapping (Weber et al 2003) Identification of these QTLs is important for the understanding of genetics of cold tolerance and can potentially be used for marker-assisted selection however application of this information for producing cold-tolerant plants will require a long time

Even though citrus is a cold-sensitive plant, citrus and its relatives can cold acclimate when they are exposed to low non-freezing temperatures. The cold acclimation process increases the freezing tolerance by inducing changes in carbohydrate metabolism

protein, lipid, and water content of cells in acclimated plants (Yelenosky 1985). An increasing level of carbohydrates and decreasing water content were observed in sweet orange [*Citrus sinensis* (L.) Osbeck cv. Valencia] grafted on cold hardy trifoliate orange [*Poncirus trifoliata* (L.) Raf.)] during cold acclimation and these changes were correlated with an increasing level of cold tolerance (Yelenosky 1992). When the water content and soluble sugar levels in field grown 'Valencia' orange (*Citrus sinensis*) were analyzed during the coolest weeks of winter, leaf water content and osmotic potential of field grown trees decreased about 20 to 25%. On the other hand, soluble sugar levels were increased by 100% resulting in an increased level of freezing tolerance (Yelenosky and Guy 1989). Exposure to low temperatures also resulted in modification of protein content in leaves of sweet orange (*Citrus sinensis* (L.) Osbeck cv. Valencia). A different polypeptide composition was observed in Valencia leaves that were exposed to 5°C for one week compared to control plants (Guy et al. 1988). It was shown that freezing tolerance of seedlings of pummelo and trifoliate orange was increased following cold acclimation. Analysis of these seedlings revealed that the polypeptide content of cold acclimated pummelo and trifoliate orange was different compared to those of nonacclimated seedlings. Although many changes were observed in trifoliate seedlings, the differences in pummelo were limited. In addition, a large polypeptide of 160 kDa was detected only in cold-acclimated trifoliate, but not in pummelo or nonacclimated controls (Durham et al. 1991).

Recent developments in molecular biology have enabled identification, isolation and characterization of cold-regulated genes and provided insight into the genetics and regulation of cold tolerance in many plants. Since the identification of the first cold

inducible genes to other plants. *Poncirus trifoliata* has been also explored for identification of cold regulated genes for improving cold tolerance in citrus. Six cold inducible cDNA sequences were isolated from a cDNA library from the leaves of cold acclimated *Poncirus trifoliata*. Further characterization of two of these cDNA sequences COR11 and COR19 revealed that these two proteins are similar to cotton D-11 and Group 2 LEA proteins. LEA proteins are expressed at high levels during embryo maturation and high stress conditions resulting from loss of intracellular water. Homologs of these genes were isolated from fruits of *Citrus unshiu* (Hara et al. 1999) and grapefruit (Porat et al. 2002).

Application of genomic techniques for studying cold response in *Arabidopsis* resulted in identification of several hundred cold-regulated genes (Seki et al. 2001, Seki et al. 2002, Fowler and Thomashow 2002). Expression of many cold-regulated genes has also been identified and characterized in other plants. Identification and characterization of these genes has helped in the understanding of mechanisms of cold acclimation and tolerance. These genes have also been used for improving cold tolerance in transgenic plants. Since citrus is cold-sensitive and cold tolerance is an important trait, understanding the cold tolerance in *Poncirus*, which is a cold-hardy citrus relative, may provide information necessary for improving cold tolerance in citrus. Although citrus and *Poncirus* have been bred for developing cold tolerance for many years, characterization of genes involved in cold tolerance has lagged behind in *Poncirus*. To date, only six cold regulated cDNAs have been identified in *Poncirus*. Considering the quantitative nature of cold tolerance in citrus and other plants, and the identification of several hundred cold regulated genes in *Arabidopsis*, a more comprehensive study is

necessary for identification of more cold regulated genes in *Poncirus*. Identification and characterization of more genes involved in cold tolerance may pave the road for developing cold-hardy citrus plants.

CHAPTER 3
IDENTIFICATION OF COLD REGULATED GENES FROM *Poncirus trifoliata* (L.) Raf. USING SUBTRACTIVE cDNA LIBRARIES

Introduction

Low temperature is one of the limiting factors for cultivation of agricultural crops in certain regions. Economically important crops have been bred for temperature tolerance for many years to improve and extend the growing regions. As for many plants, in citrus maximum freezing tolerance is not constitutive, but induced in response to non freezing temperatures below 10°C, which is called cold acclimation (Thomashow 1998). During cold acclimation, a series of physiological and biochemical changes take place in plants that lead to the induction of specific genes (Guy 1990). Changes in gene expression between cold-acclimated (CA) and nonacclimated (NA) plants have been studied extensively in many plants using differential screening of cDNA libraries (Gilmour et al. 1992, Chauvin et al. 1993, Monroy et al. 1993, Weretilnyk et al. 1993), differential display (Horvath and Olson 1998) and subtractive cDNA libraries. A number of cold-regulated genes have been identified and characterized in many plants, including *Arabidopsis* (Gilmour et al. 1992, Horvath et al. 1993), *Brassica napus* (Weretilnyk et al. 1993) *Hordeum vulgare* (Dunn et al. 1991), *Medicago sativa* (Monroy et al. 1993), and *Spinach oleracea* (Neven et al. 1993). In addition, the regulatory sequences containing CRT/DRE elements have been characterized from some of these genes (Baker et al. 1994, Yamaguchi-Shinozaki and Shinozaki 1994). A transcription

factor the CRT/DRE binding factor 1(CBF1), involved in regulation of the cold response pathway and the expression of these genes by binding to CRT/DRE element has also been isolated in *Arabidopsis* (Stockinger et al 1997, Gilmour et al 1998 Liu et al 1998), as has its homologs in *Arabidopsis* and other plants (Jaglo et al 2001). More recently microarray analysis has been developed and used for a global examination of expression of thousands of genes simultaneously under different experimental conditions (Moody 2001). Microarray analysis of thousands of genes in CA and NA *Arabidosis* showed that up to several hundred genes were regulated by low temperature. A majority of these genes (about 75%) was cold-induced and expression of others (about 25%) was repressed by cold (Seki et al 2001, Seki et al 2002, Fowler and Thomashow 2002) Fowler and Thomashow (2002) also demonstrated that only a portion of cold-regulated genes are activated by the CBF pathway, indicating that multiple regulatory pathways are involved in the expression of cold regulated genes Therefore, the identification and characterization of more genes in different plants will provide better understanding of cold response pathways and improve cold hardiness in plants

Citrus is one of the most economically important fruit crops in the world, grown commercially in almost every country in tropical and subtropical regions Cultivation of citrus is mainly limited by low temperatures inside of this region. Low temperatures and freezes also result in significant damage and economic losses in subtropical citrus growing regions Therefore, cold hardiness is a desirable trait for introduction into commercial citrus varieties. Most commercially important varieties of citrus are not cold- tolerant and are susceptible to freezes. Yet, there is significant variation among citrus species and relatives for cold tolerance ranging from very cold-sensitive types,

such as *Citrus grandis* (pummelo) to the cold-hardy interfertile citrus relative *Poncirus trifoliata* which can withstand temperatures of -26°C when cold-acclimated. Once *Poncirus trifoliata* was recognized as cold-hardy, in the previous century it was used in breeding programs mainly in Florida ((Soost and Cameron 1975) and other countries such as Japan and Russia (Gmitter et al. 1992) in efforts to produce cold tolerant commercial citrus varieties. Although cold tolerant rootstocks were produced by crossing *Poncirus* and *Citrus*, production of scion varieties with good fruit quality and cold hardiness has been unsuccessful to date, mainly because the tree and fruit characteristics of most of the crosses were undesirable as the fruit contained high levels of poncirin which gives a bitter taste. In addition, biological problems associated with citrus breeding including long juvenility periods, polyembryony, heterozygosity, sterility, self- and cross- incompatibility, inbreeding depression, and the quantitative inheritance of cold tolerance have also been limiting factors for producing cold-hardy citrus varieties (Soost and Cameron 1975, Soost and Roose 1996). Use of genomics and molecular biology techniques such as gene cloning, gene manipulation, and genetic transformation can overcome problems associated with breeding and provide new approaches for understanding and improving cold tolerance in citrus. This requires availability of genes that can be used in genetic transformation for improving cold tolerance in Citrus. To isolate such genes, cDNA libraries were previously constructed from CA and NA *Poncirus trifoliata*. Differential screening of these libraries yielded a few cold-induced genes including pBCORc115, pBCORc119, pBCORc720, pBCORc410, pBCORc102 and pBCORc510 (Cai et al. 1995). In the present study, more cold regulated genes were identified by construction and sequencing of subtractive cDNA libraries from cold-

acclimated and nonacclimated *Poncirus* seedlings Cold regulation of these genes was demonstrated by expression analysis using reverse northern and northern blot analyses in cold-acclimated and nonacclimated *Poncirus*

Materials and Methods

Plant Materials

Seeds were extracted from *Poncirus trifoliata* cv Rubideaux fruits grown in the experimental orchard of the Horticultural Sciences Department at the University of Florida, Gainesville, Florida. The seeds were planted in a soilless medium in 2" x 10" containers' in racks of twenty and seedlings were grown and maintained in the greenhouse.

Cold Acclimation for Library Construction

One-year-old seedlings with two-month-old flushes were transferred from the greenhouse to a controlled environment growth chamber for two weeks under a 16-h light/8-h dark photoperiod at 28°C Control plants were maintained under the same conditions For cold acclimation plants were transferred to another growth chamber equipped with low temperature control managed by the GEC i34S Precision Temperature Measurement and Control System (Gaffney Engineering Gainesville, Florida) which measures the chamber temperature with 13 thermocouples at different locations in the chamber These plants and maintained at 4°C under a 16-h light/8-h dark photoperiod for 2 d

Environmental Stress Treatments for Gene Expression Study

Two racks containing 40 plants of two-year-old *Poncirus* and pummelo with two-month-old flushes were first transferred from the greenhouse to a controlled environment growth chamber and maintained there for two weeks with 16 h light/8 h dark photoperiod

at 28°C. After leaf samples were collected from *Poncirus* and pummelo plants for nonacclimated and nondehydrated controls, they were subjected to cold acclimation and dehydration treatments. For cold acclimation, 20 plants from each species were transferred to another growth chamber equipped with the same low temperature control system as described above. The plants were maintained at 4°C under 16-h light/8 h dark photoperiod for seven days. Leaf tissue samples were collected from all of these plants following 1 h, 4 h, 8 h, 24 h, 2 d, 4 d, and 7 d of cold acclimation at 4°C. For dehydration, 20 plants from each *Poncirus* and pummelo were maintained in the controlled environment growth chamber with 16 h light/8 h dark photoperiod at 28°C without irrigation for one week. After one week, water continued to be withheld from the plants and the water content of the soil in each pot was measured with a time-domain reflectometry (TDR) probe. Leaf tissue samples were collected and pooled from at least four different plants with the same water content at specific time points of 7 d, 9 d, 11 d, 13 d and 15 d following dehydration. All tissue samples were immediately frozen in liquid nitrogen and stored at – 80°C until use.

Isolation of RNA

Poly A^+ RNA was isolated from leaf samples collected from at least ten individual nonacclimated and cold-acclimated plants obtained from two independent cold treatment experiments using the Fast Tract 2.0 Kit for isolation of mRNA (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions. Total RNA was isolated from leaf samples collected and pooled from at least ten individual nonacclimated and cold-acclimated plants obtained from another independent cold treatment experiment using Trizol (Gibco BRL, Rockville, MD) according to the manufacturers' instructions.

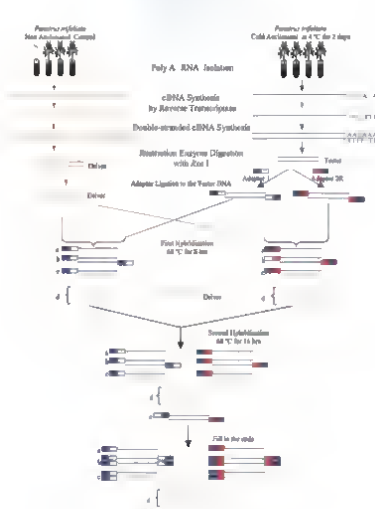


Figure 3-1 Construction of cold-regulated cDNA libraries using a subtractive hybridization method (Adopted and modified from Clontech)

Construction of the Subtractive Library

Forward and reverse subtracted cDNA libraries were established with 2 µg of Poly A+ RNA from 2 d cold-acclimated and nonacclimated Poncirus seedlings using a PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions (Figure 3-1). Subtracted cDNAs were amplified and cloned into the pT-ADV TA cloning vector using the Advantage PCR Cloning Kit (Clontech, Palo Alto, CA). Plasmids were isolated from selected clones using a 96-well plate plasmid purification kit (QIAGEN, Hilden, Germany).

Sequence analysis

Randomly chosen cDNA clones from forward and reverse subtracted libraries were sequenced using universal primers. Nucleotide sequences and deduced amino acid sequences of these clones were compared against the GenBank database using BLASTN and BLASTX, respectively.

Reverse Northern Blot Analysis of Subtracted cDNAs

cDNA clones and two negative controls (1R and 2R sequences from the human genome provided with the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA), were amplified by PCR using NP1 (5'- TCGAGCGGCCGCCCGGGCAGGT-3') and NP2 (5'-AGCGTGGTCGCGGGCGAGG T-3) primers complementary to regions flanking both sites of the cDNA inserts and Advantage DNA Polymerase Mix. A PCR reaction was performed at 94°C for 30 s, 95°C for 30 s, and 68°C for 3 min for 23 cycles according to manufacturer's instruction (Clontech, Palo Alto, CA). In addition, two previously characterized cold-induced genes, cor11 (GenBank accession number L39005) and cor19 (GenBank accession number L39004), and a constitutively expressed

atpB gene encoding β subunit of chloroplast ATP synthase from *Poncirus trifoliata* (GenBank accession number: AJ238409) were amplified using gene specific primers as positive and internal controls, respectively. PCR products of cDNAs and controls were denatured with 0.4 N NaOH and blotted twice onto a Hybond N+ nylon membrane (Amersham Biosciences) using a Bio-Dot Microfiltration Apparatus (Bio-Rad, Richmond, CA) according to the manual. This entire process was repeated to produce two identical copies of each blot.

The duplicate blots were pre-hybridized with PerfectHyb buffer (Sigma, St. Louis) with 0.1 mg/ml denatured salmon sperm DNA at 65°C for one hour and hybridized with ^{32}P-labeled single-strand cDNA probes produced by reverse transcription of 250 ng poly A$^+$ RNA isolated from cold and non-acclimated plants with an oligo-dT primer at 65°C for 16 h. Blots were washed twice with 2 X SSC, 1% SDS at 65°C for 20 min, followed by two washes with 0.2 X SSC 0.5% SDS at 65°C for 20 min each. Blots were then exposed to a phosphor screen for 16 h, scanned by the storm phospho-imaging system (Amersham, Uppsala, Sweden) and quantitated using ImageQuant (Amersham, Uppsala, Sweden). Reverse northern blots were repeated three times using cDNA probes prepared from poly A$^+$ RNA samples of two independent experiments.

Data Analysis

Data was collected from two independent reverse northern blot hybridizations containing all cDNA samples and controls in duplicate membranes each containing two spots of the each individual sample. Values of duplicate spots for each individual sample were determined after background subtraction. Since the reverse northern blot hybridizations were done on multiple membranes at different times and the experiment

was repeated three times, values for each sample were normalized using ATP synthase as the constitutive control in order to compare the data sets Normalization was done by dividing the values of each sample with the value of the constitutive control on the same blot. The ratio of the normalized data for individual genes in cold-acclimated and nonacclimated blots was used to determine fold induction of each clone. Log_{10} values of the normalized data were then used to determine the statistical differences in expression of each clone with cold and non-acclimated probes by a t-test. Clones showing more than a 2-fold change in expression and $p \leq 0.05$ were considered to be cold-regulated.

Northern Blot Hybridization

Total RNA samples from cold-acclimated and nonacclimated *Poncirus* and pummelo plants were separated on a denaturing agarose gel, transferred to a nylon membrane and prehybridized for 30 min and hybridized with Digoxigenin (DIG)-labeled DNA probes prepared by PCR labeling or DIG-labeled antisense RNA probes prepared by *in vitro* transcription at 50°C (for DNA probes) or 68°C (for RNA probes) for 16 h according to the DIG Application Manual for Filter Hybridizations (Roche Molecular Biochemicals Mannheim, Germany) The membrane was washed two times with 2 X SSC and 0 1% SDS at room temperature for 5 min, followed by two washes with 0 1 X SSC and 0 1% SDS at 50°C (for DNA probes) or 68 C (for RNA probes) for 15 min and subjected to DIG labeled DNA probes or DIG labeled RNA probes to detect RNA targets on a northern blot using the DIG Chemiluminescent Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany) Membranes were also hybridized with an 18S ribosomal RNA (rRNA) probe for loading and transfer control

Results

To identify cold-regulated genes in *Poncirus*, reverse and forward subtracted cDNA libraries were prepared using cold acclimated and nonacclimated *Poncirus* seedlings. PCR amplification of selected clones demonstrated that the libraries contained fragments ranging from 250 to 1000 bp. A total of 192 randomly picked colonies, 136 from forward (clone number starting with C, cold-induced) and 56 from reverse (clone number starting with N, cold-repressed) subtracted libraries were sequenced. The nucleotide and deduced amino acid sequences were compared using BLASTN and BLASTX, respectively. The sequence analysis revealed that a number of cDNA clones showed homology to previously characterized cold response genes in other plants including *Arabidopsis*, tobacco, tomato and potato. Cold response genes identified in this study have homology to different groups of genes, including transcription factors and DNA binding proteins, heat shock proteins, late embryogenesis proteins and some metabolic genes (Table 3-1).

To study differential expression in cold-acclimated and nonacclimated plants, these cDNA clones were analyzed by reverse northern blot hybridization. Two previously characterized cold induced genes from *Poncirus*, cor11 and cor19, and two nonhomologous genes from the human genome were included in the blot as positive and negative controls respectively to confirm the validity of reverse northern analysis. In addition, a housekeeping gene chloroplast ATP synthase from *Poncirus* whose expression is not affected by cold acclimation, was also included as an internal control to compare and analyze different blots. Reverse northern blot analysis showed that expression of both COR genes was strongly induced by cold acclimation as expected but their expression was minimal in the non-acclimated control. On the other hand, no

Figure 3-2. Differential screening of forward and reverse subtracted cDNA libraries of cold acclimated *Poncirus* by reverse northern analysis. PCR amplified cDNAs from forward and reverse subtracted libraries were spotted onto duplicate blots. Blots were hybridized differentially with ^{33}P label cold acclimated and nonacclimated cDNA probes. The Roman numerals indicate the duplicate blots. Capital letters and numbers are used for rows and columns, respectively to identify the location of individual cDNAs in each blot. The location of the water control, cor11 cor19 positive controls, 1R and 2R negative controls, and the ATP synthase constitutive control are indicated by black, red, blue and green boxes, respectively. Blots I-III contain cDNAs only from the forward subtracted library. The blot V contains cDNAs only from the reverse subtracted library. In blot IV, rows A C and E G contain cDNAs from forward subtracted library, but rows D and H contain cDNAs from the reverse subtracted library.

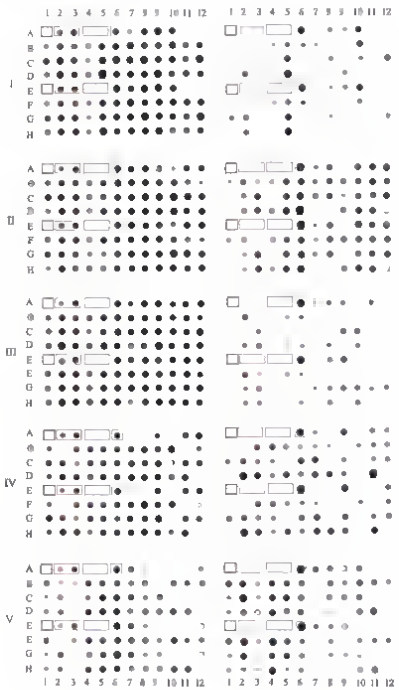

Cold Acclimated
Non Acclimated
1 2 3 4 5 6 7 8 9 10 11 12
A B C D E F G H
I
II
III
IV
V

Table 3-1 Summary of sequence and expression analysis of cold-regulated genes identified from cold acclimated *Poncirus* cDNA libraries. Significance of gene expression ratios was determined by a t-test at p ≤ 0.05

Clone[a] number	Location on the blot[b]	GenBank Match[c]	E value[d]	Fold induction[e]	t-test p-value[f]
Transcription					
C6	I-B6, F6	CCHH finger protein 4 *Arabidopsis thaliana*	0.26	22	0.001
C12	I-H12, L12	Putative bZIP Transcription Factor *Arabidopsis thaliana*	5e-18	34	0.008
C17	I-C5, G5	AP2 domain protein homolog *Tomato*	8e-33	3	0.05
C32	I-D8, H8	Gene for glycine-rich RNA-binding protein *Citrus unshiu* (2)	e-112	10	0.01
C34	I-D10, H10	Putative DNA binding protein (RAV2-like) *Arabidopsis thaliana*	6e-21	11	0.02
C82	III-B6, F6	DNA-binding protein WRKY1-parsley	4e-21	5	0.00004
Cell rescue, defense, cell death and aging					
C5	I-B5, F5	Wound-induced protein (WI12) mRNA *Mesembryanthemum crystallinum*	1e-11	4	0.02
C7	I-B7, F7	COR(c)115 cold stress protein mRNA *Poncirus trifoliata* (5)	4e-38	48	0.0003
C22	I-C10, G10	Hsc70 mRNA for heat shock protein 70 cognate *Salix gilgiana* (3)	5e-94	10	0.008
C33	I-D9, H9	Late Embryogenesis protein homolog *Tomato* (7)	3e-11	19	0.006
C36	I-D12, H12	DHN1 cold regulated LTCOR10 *Populus euramericana*	5e-08	10	0.01
C42	II-B6, F6	COR(c)119 cold stress protein mRNA *Poncirus trifoliata*	1e-38	15	0.003
C44	II-B8, F8	Elicitor inducible protein *Nicotiana tabacum*	9e-17	7	0.01
C58	II-C6, G6	Putative leucine rich protein *Oryza sativa*	7e-23	4	0.05
C63	II-C11, G11	Late embryogenesis abundant protein 5 mRNA *Nicotiana tabacum* (2)	0.003	8	0.01
C64	II-C12, G12	bdr1 mRNA *Vigna unguiculata* (2)	2e-10	10	0.001
C72	II-D8, H8	Seed maturation protein PM39 *Glycine max*	3e-11	5	0.03
C119	III-A7, E7	High molecular weight heat shock protein (Hsp81) *Malus domestica*	5e-87	2	
C120	III-A8, E8	Avr9/Cf-9 rapidly elicited protein 65 *Nicotiana tabacum*	7e-05	3	0.05
Cellular communication and signal transduction					
C9	I-B9, F9	Nine-cis-epoxycarotenoid dioxygenase *Tomato*	6e-19	22	0.0005
C21	I-C9, G9	ACC oxidase *Citrus sinensis*	e-129	36	0.0003
C40	II-B4, F4	Arginine decarboxylase *Vitis vinifera* (2)	5e-08	8	0.03
C78	III-B2, F2	Putative RING zinc finger protein *Arabidopsis thaliana*	5e-28	9	0.0001
C94	III-C6, G6	Putative ripening-related protein *Vitis vinifera*	8e-37	2	0.08
C96	III-C8, G8	Phosphoinositide kinase *Pisum sativum*	3e-93	4	0.01
C117	III-A1, E1	Putative protein kinase *Oryza sativa*	0.48	3	0.01
Metabolism					
C3	I-B3, F3	NADP-malate dehydrogenase mRNA *Citrus limon*	e-110	10	0.003
C27	I-D3, H3	Nitrate reductase (2)	5e-21	17	0.0002
C61	II-D1, H1	rolA/rolB-like protein RSHI *Nicotiana tabacum*	5e-62	7	0.03
C71	II-D7, H7	Isoflavone reductase related protein *Pyrus communis*	2e-82	4	0.03
C79	III-B3, F3	Putative protein translation factor	1e-71	8	0.0003
C87	III-B11, F11	Hydroxymethylglutaryl coenzyme A synthase *Hevea brasiliensis*	9e-89	3	0.01
C100	III-C12, G12	Rieske FeS precursor protein mRNA for Spinach	1e-83	5	0.0007
C107	III-D7, H7	mRNA for 1-deoxyxylulose 5-phosphate synthase *Catharanthus roseus*	2e-14	8	0.03
C113	II-A7, E7	Glutathione S-transferase *Euphorbia esula*	2e-18	4	0.04
C122	III-A10, E10	Aminotransferase	4e-66	3	0.0002
C133	IV-B9, F9	Thiamin biosynthesis protein *Citrus sinensis*	4e-85	5	0.01
Transport facilitation					
C8	I-B8, F8	ABC1 protein *Nicotiana plumbaginifolia*	4e-09	10	0.1
C88	III-B12, F12	Hexose carrier (HexB) mRNA *Ricinus communis*	6e-57	4	0.0001
C108	III-D8, H8	Putative ABC transporter *Arabidopsis thaliana*	3e-10	6	0.01
Energy					
C61	II-C9, G9	Phosphoenolpyruvate carboxykinase *Flaveria pringlei*	3e-24	4	0.03
C121	III-A9, E9	fructose-bisphosphate aldolase 1-like protein *Arabidopsis thaliana*	7e-60	9	0.008
Cellular biogenesis					
C60	II-C7, G7	Plasma membrane protein *Arabidopsis thaliana*	9e-10	13	0.0009
C75	II-D11, H11	Similar to glucose-6-phosphate/phosphate translocator *Arabidopsis thaliana*	3e-21	5	0.02

Table 3-1 Continued

Clone[a] number	Location on the blot[b]	GenBank Match[c]	E value[d]	Fold induction[e]	t-test p-value[f]
	Protein fate				
C74	II-D10, H10	ATP-dependent Clp protease (CD4B) *Lycopersicon esculentum*	8e-12	5	0.01
C90	III-C3, G3	Ubiquitin-conjugating enzyme	3e-06	13	0.000005
C1	I-B1, F1	Alpha-crystallin-related protein mRNA *Trichoderma mentagrophytes* (5)	8e-06	0	0.01
	Unknown role				
C10	I-C7, C7	Putative MSN5-like protein mRNA *Dianthus caryophyllus* (5)	5e-04	18	0.0006
C43	II-G7, F7	Metallopanstimulin mRNA *Strongyloides ratti* (4)	1e-09	0	0.01
	Proteins with unknown function				
C4	I-B4, F4	Unknown protein		7	0.02
C10	I-B10, F10	Unknown protein		7	0.07
C16	I-C4, G4	Unknown protein		12	0.01
C28	I-D4, H4	Unknown protein		7	0.01
C35	II-D11, H11	Putative protein		5	0.008
C39	II-E3, F3	Unknown Protein		14	0.001
C49	I-A7, E7	Unknown protein		0	0.01
C53	II-C1, G1	Unknown protein		7	0.01
C62	II-C11, G11	Unknown protein		7	0.007
C66	II-D2, H2	Hypothetical protein		9	0.009
C76	II-D12, H12	Unknown protein		7	0.02
C77	III-B1, F1	Unknown protein		0	0.0006
C83	III-B7, F7	Unknown Protein		7	0.002
C95	III-C7, F7	Unknown Protein		6	0.01
C98	III-C11, F11	Unknown Protein		2	0.03
C110	III-D1, H1	Unknown Protein		10	0.001
C100	III-D9, H9	Unknown Protein		5	0.002
C123	III-A11, E11	Unknown Protein		2	0.003
C128	IV-B5, F5	Unknown Protein		2	0.03
C130	IV-B6, F6	Unknown Protein		4	0.008
N8	IV-A8, E8	Unknown Protein		3	0.01

[a] C indicates cold induced and N indicates cold repressed cDNA

[b] The location of the individuals cold regulated clones on the reverse northern blot shown in Figure 3-2

[c] The GenBank sequences showing the highest nucleotide and/or amino acid sequence homology with the individual cDNA clones. The numbers in the parentheses indicate the number of clones showed homology with the specific sequence in the databases.

[d] The Expect (E) value is a parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size

[e] Fold induction indicates the ratio of the normalized values of individual genes obtained from two independent reverse northern blots with cold- and non-acclimated cDNA probes

[f] The statistical differences in expression of each clone was determined by comparison log_{10} values of the normalized data from cold and non-acclimated probes by t test. The clones showing at least 2-fold change in expression and $p < 0.05$ for t test are shown

expression of the human genes, 1R and 2R, was detected. Although no difference was observed in the level of expression of ATP synthase, visible differences were observed in the expression levels of most cDNAs from the forward subtracted library in the cold-acclimated and nonacclimated blots (Figure 3-2).

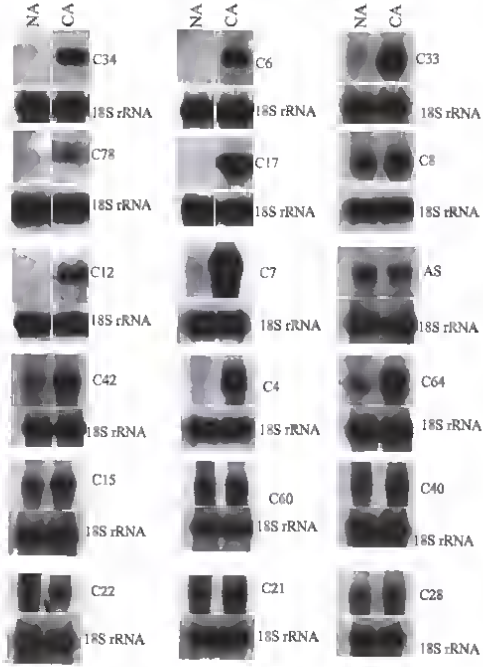
Statistical analysis of the reverse northern blots demonstrated that 97 of 192 clones showed differential expression in cold-acclimated and nonacclimated conditions. Differential expression of cDNAs was detected by fold changes in their expression in two independent experiments and confirmed with t-tests ($p \leq 0.05$). Among the 97 cold-regulated cDNA clones, 96 were induced and only one cDNA was repressed by cold acclimation. Of the 96 cold-induced cDNAs, 76 showed homology with genes with known function(s) in plants or other organisms; however, 20 cDNAs showed homology with genes of unknown function or did not show homology with any previously sequenced genes in GenBank. The expression level of the cold-induced genes ranged from two to 49-fold, indicating that different cDNAs were induced at different levels. Two of the genes identified in this study were identical to previously identified cold response genes in *Poncirus*, cor11 and cor19, indicating that our subtractive library and reverse northern analysis functioned properly to identify cold-inducible genes. Newly identified cold-inducible genes in *Poncirus* showed homology to cold-regulated genes encoding functional proteins in *Arabidopsis* and other plants including late embryogenesis abundant proteins (LEA), heat shock proteins, dehydrins, and those involved in signal transduction, cell defense, metabolism, transport facilitation, and cellular biogenesis. In addition, cDNAs showing homology with previously identified transcription factors involved in environmental stress response in *Arabidopsis* such as

bZIP, zinc finger, RAV2-like, WRKY1, and AP2 domain containing proteins were identified in *Poncirus*. The complete list of cold-induced genes and their possible functions are shown in Table 3-1. The expression level of the cold-repressed gene was reduced 3 fold during cold acclimation. This gene did not show homology with a protein of known function (Table 3-1).

To confirm differential expression of cold-regulated genes identified by reverse northern analysis, 17 cold induced cDNAs including C6, C7, C8, C9, C12, C15, C17, C21, C22, C28, C33, C34, C40, C42, C60, C64, and C78 were selected for northern blot analysis. Total RNA from two-day cold-acclimated and nonacclimated plants was analyzed for expression of these genes and ATP synthase using gene specific probes. Northern blot analysis demonstrated that all cold induced genes were expressed differently in cold acclimated and nonacclimated plants. The difference in the expression of cold-inducible genes was not due to differences in amount of RNA or experimental variation since the 18S RNA was similar and no significant change in expression of ATP synthase was observed in both cold-acclimated and nonacclimated plants.

In northern blot analysis C7, which is homologous to the previously characterized cor19 gene in *Poncirus*, showed the highest level of expression. This result was consistent with reverse northern analysis where its expression increased 49 fold in cold-acclimated plants. A number of other cDNAs including C12, C60, C8, C33, C78, C15, C64, C34, C42, C22, C4, and C40 showed little or no expression in nonacclimated plants; however, their expression levels were highly induced in 2 d cold-acclimated plants (Figure 3-3). High levels of expression of these genes in northern blots were correlated with their fold induction in reverse northern blot analysis. On the other hand, expression

Figure 3-3 Northern blot analysis of selected cDNAs from the cold-induced library Total RNA from 2 d cold acclimated (CA) and nonacclimated (NA) *Poncirus* seedlings were separated on a denaturing agarose gel and blotted onto a nylon membrane Blots were hybridized with DIG-labeled DNA probes specific to individual genes followed by DIG- labeled 18S rRNA probe for loading and transfer control The cDNAs show homology with gene encoding following proteins. C34 RAV-like protein C78 RING zinc finger protein C12 bZIP protein C42 Sodium antiporter C15 Nitrate Reductase, C22 Heat shock protein 70 C06 CCHH finger protein, C17 AP2 domain protein C07 Cor19 homologue dehydrin, C04 Unknown protein, C60 Peroxisomal membrane protein, C21 ACC oxidase, C33 LEA homolog protein, C08 ABC1 transporter protein AS ATP synthase, C64 bdn1 homologue dehydrin, C40 Arginine Decarboxylase, C28 Unknown protein


NA
CA
C34
18S rRNA
C78
18S rRNA
C12
18S rRNA
C42
18S rRNA
C15
18S rRNA
C22
18S rRNA
NA
CA
C6
18S rRNA
C17
18S rRNA
C7
18S rRNA
C4
18S rRNA
C60
18S rRNA
C21
18S rRNA
NA
CA
C33
18S rRNA
C8
18S rRNA
AS
18S rRNA
C64
18S rRNA
C40
18S rRNA
C28
18S rRNA

of some cDNAs including C60, C21 and C28 were detected in nonacclimated and cold acclimated plants by northern blot analysis but expression of these cDNAs was slightly higher under cold acclimation. Although differences in expression can be detected under different conditions, levels of expression were not correlated with their fold induction in reverse northern blot analysis. Only one cDNA, C17, which was induced only three-fold on reverse northern blots, showed little expression in nonacclimated plants, but very high expression in cold-acclimated plants on northern blots.

Among the cold induced genes, C12, which is homologous to a bZIP transcription factor from *Arabidopsis*, was selected for further study. This cDNA was induced 24 fold in reverse northern blot analysis and its expression was confirmed by northern blot analysis to 2 d cold acclimated and nonacclimated *Poncirus* plants. C12 expression at different time points following cold and drought treatments was studied in cold-hardy *Poncirus* and cold sensitive pummelo. Northern blot analysis revealed that in cold-hardy *Poncirus*, expression of this gene was induced at 4 h, reached peak level at 2 d, and remained at this high level at 7 d of cold acclimation (Figure 3-4). On the other hand, only slight induction of expression was observed in cold-sensitive pummelo in response to cold starting at 8 h of cold acclimation. Based on northern blot analysis, the level of expression and induction of C12 by cold was insignificant in pummelo compared to expression in *Poncirus*. When expression of C12 was studied in dehydrated *Poncirus* and pummelo seedlings, no significant changes in expression of C12 were observed, indicating that expression of C12 is only induced in response to cold, but not dehydration.

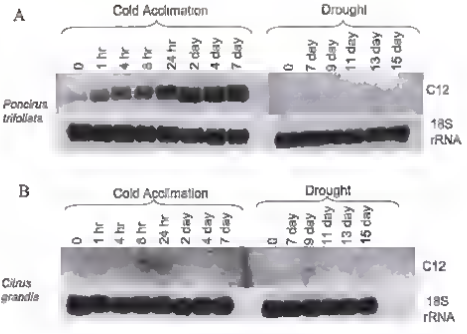


Figure 3-4 Northern blot analysis of C12 expression in response to environmental stresses (A) Expression of C12 in response to cold acclimation and drought in *Poncirus* detected by antisense DIG-labeled riboprobe. (B) Expression of C12 cDNA in response to cold acclimation and dehydration (drought) in pummelo detected by antisense DIG-labeled riboprobe. The type of environmental stress treatment and the duration of the treatment are indicated above each blot. The expression of 18 *S rRNA* was used as a loading and transfer control and is shown below the expression of the specific gene.

Discussion

It has been shown that cold hardiness is a quantitative trait involving hundreds of genes. Recent developments in genomic techniques and their application for studying cold response in *Arabidopsis* has confirmed the quantitative nature of cold hardiness by identifying several hundred cold regulated genes (Seki et al 2001, Seki *et* al 2002, Fowler and Thomashow 2002). These studies not only identified genes, but also provided insight into the regulation of these genes in response to low temperature. Although not to the same extent as in *Arabidopsis*, the expression of many cold-regulated genes has also been studied and characterized in other plants Only one previous study has been done on cold-regulated genes in *Poncirus*, which resulted in the identification of seven cDNAs and the characterization of two genes coding for group II LEA proteins (Cui et al 1995) To identify more cold-regulated genes in *Poncirus*, our study used the subtractive hybridization method for construction of cold-regulated cDNA libraries

Expression of 192 cDNAs was analyzed in cold-acclimated and nonacclimated *Poncirus* using reverse northern blot analysis In these experiments, several control strategies were used to ensure the validity of results. Plant materials from which RNA was isolated were pooled from at least ten similarly treated individual plants to eliminate biological variation Thus, the changes in gene expression reflect the common response of all plants rather than the response of a single plant Two previously identified cold-inducible genes from *Poncirus* were used as positive controls, and two human genes with no homology to plant genes along with a water negative control were included in expression studies by reverse northern analyses to confirm the specificity of hybridization Expression studies were repeated at least twice with two different RNA

preparations from two independent cold acclimation experiments. Each blot contained two replicates of each cDNA sample at different locations in the blot. To eliminate experimental differences in different blots and to compare results from different experiments, expression data was normalized with an internal control, ATP synthase from *Poncirus*. In addition, expression of a number of selected genes was studied by northern blot analysis of RNA from another independent cold acclimation experiment.

Sequencing and expression analysis of a small fraction (192 clones) of the forward and reverse subtracted libraries resulted in identification of a total of 97 cold regulated cDNAs in *Poncirus*. These genes included two previously characterized cold response genes, cor11 and cor19 in *Poncirus* (Cai et al. 1995) and showed homology with previously identified environmental stress response genes, especially cold response genes in *Arabidopsis* (Seki et al. 2001, Seki et al. 2002, Fowler and Thomashow 2002) and other plants including tomato, potato, rice, maize, wheat, and barley. Identification of genes homologous to previously characterized genes suggests that the subtractive hybridization and expression analysis functioned properly. Since 97 cold-regulated genes were identified from the screening of only 192 cDNAs, sequencing and expression analysis of more cDNAs from the forward and reverse subtracted libraries could result in identification of many more cold-regulated genes. This study demonstrated that construction of a subtractive library is a useful method for identification of differentially expressed genes and that this method could be used for identification and expression analysis of novel genes under different conditions in *Poncirus*, *Citrus* and other plants which have limited sequence information.

Cold-induced genes identified in this study show homology with genes involved in a variety of cellular functions ranging from transcription to transport facilitation. The majority of genes reported here and their functions are similar to cold-induced genes identified in *Arabidopsis* by microarray analysis (Seki et al. 2001; Seki et al. 2002 Fowler and Thomashow 2002). As in *Arabidopsis* and other plants, cold-induced genes in *Poncirus* can be divided into two groups, genes encoding regulatory and functional proteins. Regulatory proteins are involved in cellular communication, signal transduction, and regulation of gene expression. A number of genes showing homology to transcription factors such as bZIP, AP2 domain, RAV2-like, WRKY1 DNA-binding and zinc finger proteins regulating expression of cold regulated genes as well as glycine rich RNA binding protein were identified. Although the number of genes encoding regulatory proteins is limited in *Poncirus* compared to *Arabidopsis*, the presence of these cold responsive transcription factors in *Arabidopsis* and *Poncirus* indicates that similar regulatory pathways are activated during cold acclimation. Since we identified genes induced at 2 d of cold acclimation and only a limited number of cDNAs was analyzed, gene expression studies at different time points and sequencing and expression analysis of more cDNAs from the cold induced library may result in the identification of more regulatory proteins.

Genes encoding functional proteins such as late embryogenesis abundant proteins (LEA), heat shock proteins, sugar metabolism and oxidative stress related proteins identified in this study may be involved in cold tolerance. LEA proteins stabilize membranes and proteins through detergent-like or chaperone activities, thus, they protect the integrity of cell (Close 1996 1997, Wisniewski et al. 1999). We identified several

cDNAs in *Poncirus* showing homology to LEA proteins in different plants indicating that *Poncirus* is expressing a similar group of genes during cold acclimation to adapt to the changes imposed by low temperature. Heat shock proteins (HSPs) act as chaperones to stabilize the proteins by refolding the denatured proteins and preventing protein aggregation (Sung et al. 2001). In response to stress conditions, HSPs are needed more and thus their expression increases in stressed plants. Two different HSPs were identified in response to cold stress in *Poncirus* which were also cold-induced in other plants. Sugars are involved in osmoregulation by increasing intracellular osmotic potential and act as cryoprotectants by protecting cell membranes and proteins (Guy 1990, Crowe et al. 1990, Crow et al. 1993). Expression of sugar transporters, such as hexose carrier and glucose-6-phosphate/phosphate translocator and enzymes involved in sugar metabolism, such as fructose bisphosphate aldolase and phoshoenolpyruvate carboxykinase were increased in cold-treated *Poncirus*. Cold stress also increases active oxygen species (AOS) which causes oxidative stress in the cell. Glutathione S-transferase (GST) is a detoxification enzyme and may have a role in protecting cells from oxidative stress. Expression of GST was reported to be induced in response to cold treatment to alleviate the effect of AOS in the cell (Seppanen et al. 2000). A cDNA homologous to GST showed increased expression in cold-acclimated *Poncirus*, suggesting that detoxification of AOS is being used by *Poncirus* to cope with oxidative stress-induced by cold treatment.

Changes in protein expression, stability and turnover were observed in response to cold treatment to plants. ATP dependent Clp protease and ubiquitin conjugating enzyme are involved in removal of damaged or inactivated proteins as well as turnover of specific

proteins to control the metabolic and developmental processes (Rochsteiner 1987 Vierstra 1993). These two proteins were induced during cold acclimation of *Poncirus* and *Arabidopsis* (Seki et al 2002 Zheng et al 2002) There is a relationship between cold response and ABA level where ABA levels increased in response to low temperature In plants (Luo et al 1992 Lång et al 1994) and exogeneous application of ABA increased the freezing tolerance of many plants (Orr et al 1986 Ishikawa et al 1990) Therefore increased expression of genes involving ABA biosynthesis and ABA signaling pathway were reported. Nine-cis-epoxycaretenoid dinxygenase (NCED), a key enzyme in ABA biosynthesis which catalyzes the conversion of 9-cis-epoxycaretenoids to xanthoxin (Iuchi et al 2001), was induced by cold in *Poncirus* In addition, a bZIP transcription factor was identified as a cold-responsive gene in this study Since the bZIP transcription factor induces gene expression through *cis*-elements that include the ABA response element (ABRE) (Jakoby et al 2002) increased expression of these genes indicates that an ABA-dependent pathway is activated during cold acclimation in *Poncirus* Ethylene is also increased when plants are exposed to cold. In this study, a cDNA showing homology to 1 aminocyclopropene-1-carboxylate (ACC) oxidase, which is responsible for converting ACC to ethylene was increased in cold-acclimated *Poncirus* In addition, increased expression of a ripening-related protein was observed in *Poncirus* indicating that cold response and ethylene response pathways may interact. Polyamines (PAs) are commonly found in plants and their levels are increased In response to stress conditions such as low temperature. Arginine decarboxylase (ADC) is the key enzyme involved in the synthesis of PAs and expression of the gene encoding ADC increases in response to environmental stresses including cold (Mo and Pua 2002)

We found that expression of ADC was increased during cold treatment in *Poncirus* and a similar result was reported in *Arabidopsis* (Seki et al 2002)

Northern blot analysis of expression of a number of selected genes identified by reverse northern blot hybridization confirmed that these genes were all induced in response to 2 d cold acclimation in *Poncirus* This shows that the reverse northern blot analysis and statistical analysis performed in this study were effective methods for identification of cold-regulated genes An expression study of one cDNA C12, possibly encoding a bZIP transcription factor in cold hardy and cold sensitive species showed that the expression of this gene is gradually increased and reached peak level at 2 d in response to cold However, no change in expression was observed in response to drought This result not only revealed the expression pattern of potentially important regulatory gene, but also suggested that genes selected by reverse northern analysis in this study are likely to be cold-regulated.

Identification of a number of cold regulated genes in this study indicated that although only a fraction of the forward and reverse subtracted libraries were sequenced and analyzed, we identified a number of genes significantly up-regulated during cold acclimation. However only one down-regulated gene was identified. This may be due to the lack of down regulated genes in response to low temperature in *Poncirus* or to screening of a limited number of clones Since we analyzed expression of only 56 cDNAs from reverse subtracted library compare to 136 cold induced, the number of cDNAs was limited. Microarray studies with 7000 and 8000 cDNAs showed that a majority (75%) of cold regulated genes was induced in response to cold in *Arabidopsis* However, only 25% of them were repressed in response to cold indicating that the

number of genes induced by cold is much higher than the repressed ones. Therefore, it is likely that analysis of limited number of clones and down regulation of fewer genes in response to cold in plants contributed to identification of a single down-regulated gene in *Poncirus*

CHAPTER 4
ISOLATION AND CHARACTERIZATION OF COLD INDUCED PUTATIVE TRANSCRIPTION FACTORS IN *Poncirus trifoliata* (L.) Raf

Introduction

During cold acclimation, a series of physiological and biochemical changes take place in plants which results from induction of specific gene expression (Guy 1990) Expression analyses of cold responsive genes have revealed that several hundreds of genes are induced during cold acclimation in *Arabidopsis* (Seki et al 2001, 2002 Flower and Thomashow 2002) These studies and others have shown that cold-induced genes are involved in a variety of different cellular functions, including transcription, metabolism, protein fate, transport facilitation, biogenesis, cellular communications and signal transduction, cell rescue and defense, cell death and aging (Seki et al 2001 and 2002, Fowler and Tomashow 2002 Hazen et al 2003) Further characterization of cold-induced genes demonstrated that some of these genes encode functional proteins involved in increased biosynthesis of compatible solutes (Sakamoto and Murata 2002) alterations of lipid composition and membrane structure (Nishida and Murata 1996) increased levels of antioxidant activity (Iba 2002) as well as biosynthesis of stress hormones, all of which lead to an increase in cold tolerance. Additionally some cold induced genes encode regulatory proteins involved in signal transduction and regulation of gene expression during cold acclimation

Since physiological and biochemical changes during cold acclimation are associated with changes in gene expression, transcriptional regulation of cold responsive

genes has been explored for a better understanding of cold response pathways and improving cold tolerance in plants. Expression of a number of transcription factors or DNA-binding proteins increase during cold treatment of *Arabidopsis* and other plants. Most of these transcription factors contain conserved DNA binding domains found in eukaryotes such as a basic-leucine zipper (bZIP), basic-helix-loop-helix (bHLH), RING zinc finger, zinc finger MYB homo box, MADS box, as well as AP2/ERF, AP2/B3 and WRKY DNA-binding motifs found in plants (Kagaya et al. 1999, Chen et al. 2002, Seki et al. 2001, 2002, Flower and Thomashow 2002; Sakuma et al. 2002).

One of the most important research developments in cold acclimation and cold response in plants was identification of the CBF/DREB1 transcription factors that regulate an ABA independent cold response pathway in *Arabidopsis* (Stockinger et al. 1997, Gilmour et al. 1998, Liu et al. 1998). The CBF/DREB1 proteins induce expression of cold- and dehydration-regulated genes containing a CRT/DRE (C-repeat/dehydration responsive element) CCGAC core sequence in their promoter region (Baker et al. 1994, Yamaguchi-Shinozaki and Shinozaki 1994). CBF/DREB-like proteins were also identified and characterized in *Brassica napus*, a close relative of *Arabidopsis* (Jaglo et al. 2001, Gao et al. 2002) and cold-acclimated wheat, rye (Jaglo et al. 2001), and barley (Choi et al. 2002) as well as tomato, which does not cold acclimate, (Jaglo et al. 2001) indicating that the CBF/DREB cold response pathway is conserved among a number of plants including cold-sensitive and nonacclimating plants (Jaglo et al. 2001, Choi et al. 2002).

Transcription factors containing an AP2/ERF domain belong to a plant specific transcription factor family with more than a hundred members. Based on the number of

AP2/ERF domains present in the protein, these transcription factors are divided into two subfamilies (Riechmann and Meyerowitz 1998 Riechmann et al. 2000) The first subfamily contains transcription factors with two AP2/ERF domains including APETALA2 (AP2) (Jofuku et al 1994) and AINTEGUMENTA (ANT) (Elliott et al 1996) from *Arabidopsis* and Glossy15 (Moose...et al 1994) from maize, all of which are involved in flower and seed development The transcription factors containing only one AP2 domain include ERF TINY DREB1/CBF, DREB2, Pus, ABI4, and many more from *Arabidopsis* and other plants (Sakuma et al 2002) These transcription factors show sequence similarity only in the AP2/ERF DNA-binding domain and they function mostly in response to biotic and abiotic stresses including cold

The other subfamily of AP2/ERF transcription factors contains one AP2/ERF and one B3 DNA-binding domain have also been identified Two of these proteins, RAV1 and RAV2, were isolated from *Arabidopsis* The B3 domain in these proteins shows homology to the DNA binding domain in VIVIPAROUS1 (VP1) from maize (Suzuki et al 1997) and its ortholog ABI3 in *Arabidopsis* (Giraudat et al 1992). The AP2 and B3 domains of RAV1 bind independently to CAACA and CACCTG *cis*-elements respectively, and the presence of both domains increases the binding activity and specificity of the protein (Kagaya et al. 1999) Recently, it was shown that the RAV1 DNA-binding protein was induced in response to cold, suggesting that it is involved in cold induced transcriptional activation in *Arabidopsis* (Fowler and Thomashow 2002)

RING (Really Interesting New Gene) zinc finger domain containing proteins are another group of regulatory proteins. The RING finger domain is involved in zinc-binding and it has a cysteine rich sequence defined by the consensus sequence of

$CX_2CX_{(8-39)}CX_{(1-3)}HX_{(2-3)}CX_2CX_{(4-48)}CX_2C$ where X can be any amino acid. The RING finger proteins can be divided into two groups: RING-HC and RING-H2, based on the presence of Cys or His at the fifth coordination site, respectively (Freemont 2000; Jonzeiro and Weissman 2000). This domain is found in diverse proteins from a variety of species ranging from viruses to eukaryotes, including animals, yeast, and plants and is involved in a variety of cellular functions, including oncogenesis, viral gene expression, signal transduction, peroxisome biogenesis, DNA repair and recombination, and membrane vesicle sorting (Borden and Freeman 1996, Saurin et al. 1996). The function of RING zinc finger proteins was not clear until recently. It has now been demonstrated that they are involved in specific ubiquitination of proteins through a ubiquitin-dependent proteolysis pathway (Freemont, 2000, Jonzeiro and Weissman 2000). Since this pathway regulates the specific degradation of a large number of proteins involved in diverse cellular processes including cell cycle regulation, signal transduction, metabolic regulation, cell differentiation, and stress responses (Hershko and Ciechanover 1998), RING zinc finger proteins may have important roles in these processes.

A number of RING finger proteins were identified in the *Arabidopsis* genome, however, no function has been assigned for the majority of these genes (Jensen et al. 1998). Only a few plant RING zinc finger proteins have been thoroughly characterized, including COP1 and HOS1 which are negative regulators of photomorphogenesis in the dark (von Arnim and Deng 1993, Torii et al. 1999) and cold responsive gene expression (Lee et al. 2001) in *Arabidopsis*, respectively. Several other plant RING zinc finger proteins involved in seed development (Zou and Taylor 1993, Molnar et al. 2002, Lechner

et al 2002) and pathogen defense (Takai et al 2002, Gao et al 2003) have been isolated recently and partially characterized

Since some transcription factors and other regulatory proteins are cold induced and play important roles in cold-responsive gene expression, we selected two cDNAs showing homology with AP2/ERF family proteins and one with a RING zinc finger protein homology from a cold-induced subtracted cDNA library of 2 d cold-acclimated *Poncirus trifoliata* For further characterization the full-length cDNAs were isolated by random amplification of cDNA ends (RACE) and expression of these genes was studied in response to cold and drought stress in *Poncirus* and *Citrus*

Materials and Methods

Plant Materials

Poncirus trifoliata cv Rubidoux and *Citrus grandis* cv DPI 6-4 (Pummelo) seeds were extracted from trifoliate orange fruits produced in the experimental orchard of the Horticultural Sciences Department at the University of Florida, Gainesville, Florida and pummelo fruits produced in the experimental orchard of University of Florida Citrus Research and Education Center in Lake Alfred Florida, respectively The seeds were planted in a soilless medium in 2" x 10" conetainers in racks of twenty and seedlings were grown and maintained in the greenhouse

Environmental Stress Treatments

Two racks containing 40 plants of two-year old *Poncirus* and pummelo with two-month old flushes were first transferred from the greenhouse to a controlled environment growth chamber and maintained there for two weeks with 16 h light/8 h dark photoperiod at 28°C After leaf samples were collected from *Poncirus* and pummelo plants for non-acclimated and non-dehydrated controls, they were subjected to cold acclimation and

dehydration treatments. For cold acclimation 20 plants from each species were transferred to another growth chamber equipped with low temperature control managed by the GEC134S Precision Temperature Measurement and Control System (Gaffney Engineering, Gainesville, Florida). The plants were maintained at 4°C under 16-h light/8-h dark photoperiod for seven days. Leaf tissue samples were collected from all of these plants at 1 h, 4 h, 8 h, 24 h, 2 d, 4 d, and 7 d of cold acclimation at 4°C. For dehydration, 20 plants from each *Poncirus* and punmelo were maintained in the controlled environment growth chamber with 16 h light/8 h dark photoperiod at 28°C without irrigation for one week. After one week, water continued to be withheld from the plants and the water content of the soil in each pot was measured with a time-domain reflectometry (TDR) probe. Leaf tissue samples were collected from at least four different plants with the same water content at specific time points of 7 d, 9 d, 11 d, 13 d and 15 d of dehydration. All tissue samples were immediately frozen in liquid nitrogen and stored at −80°C until use.

Isolation of RNA

Total RNA was isolated from the various leaf samples using Trizol Reagents (Gibco BRL, Rockville, MD) according to the manufacturer's instructions. Total RNA concentration was determined spectrophotometrically and the samples were stored at −80°C until used.

Rapid Amplification of cDNA Ends (RACE)

The partial sequences of the selected cold-induced cDNAs were used for designing reverse and forward primers to obtain the 5' and 3' ends of the full-length cDNAs, respectively. Total RNA from 2 d cold-acclimated *Poncirus* seedlings was used as

template for generating the 3' and 5' RACE ready cDNAs for PCR amplification. The RACE ready cDNAs were used with gene specific reverse and forward primers to obtain 5' and 3' end sequences of the specific genes. All steps of RACE were performed using the Smart RACE cDNA Amplification Kit according to the manufacturer s instructions (Clontech, Palo Alto, CA)

Sequence Analysis

The partial sequences of cold-induced cDNAs obtained from the subtractive library and the 3' and 5' sequences obtained from RACE were assembled and aligned to determine full-length sequences. The assembled sequences were analyzed for open reading frames using Vector NTI suite (InforMax, Frederick, MD) The full-length cDNA sequences and the deduced amino acid sequences were then compared with previously characterized DNA and protein sequences in GenBank. The conserved domains within the protein sequences of cold-induced cDNAs were detected by a conserved domain search and compared with proteins containing the same or similar domain(s)

Northern Blot Hybridizations

Total RNA samples from environmental stress-treated and control *Poncirus* and pummelo plants were separated on denaturing agarose gels and transferred to nylon membranes The membranes were prehybridized at 68 C for 30 min and hybridized with DIG-labeled antisense RNA probe specific to the 3' half of the individual genes prepared using a DIG RNA labeling kit (Roche Molecular Biochemicals- Mannheim, Germany) at 68°C for 16 h. The membranes were then washed two times with 2 X SSC and 0 1% SDS at room temperature for 5 min, followed by two washes with 0 1 X SSC and 0 1% SDS at

68°C for 15 min and subjected to detection of DIG-labeled RNA probes to detect RNA targets on northern blots using the DIG Chemiluminescent Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany). After hybridizations with gene specific probes, the membranes were also hybridized with an 18S ribosomal RNA (rRNA) probe for loading and transfer control.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

cDNA was synthesized from 1.5 μg total RNA by Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) using oligo-dT primer according to manufacturer's instructions. PCR amplification was conducted in a 50 μl reaction mixture containing PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1% Triton X-100], 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 50 pmole gene specific primers, 2.5 U *Taq* DNA polymerase (Promega, Madison, WI) and 1 μl cDNA template. The amplification reaction was carried out in a PTC-100 Thermocycler (MJ Research, Waltham, MA). The thermocycler was programmed at 94°C for 5 min initial denaturation for one cycle and 25 or 35 cycles at 94°C for 30 s denaturation, 55°C for 30 s primer annealing, 72°C for 1 min primer extension followed by one cycle of final primer extension at 72°C for 10 min. PCR products were separated in 1.5% agarose gel by electrophoresis in TAE buffer [0.5 M Tris-Base, 0.5 M EDTA (pH 8.0), 12.6% glacial acetic acid]. Products were stained with ethidium bromide and visualized and photographed under UV light using the IS-1000 digital gel imaging system (Alpha Innotech, San Leandro, CA).

Results

Cold-Induced PI-B05 cDNA from Poncirus Encodes an AP2 Domain Containing Protein

The partial cDNA sequence of PI B05 (C17) was isolated from a subtractive cDNA library of 2 d cold acclimated *Poncirus*. Reverse northern analysis revealed that PI-B05 showed three-fold induction in response to cold and partial deduced amino acid sequences of this cDNA showed homology with AP2 domain containing proteins from other plants. Since a number of AP2 domain containing proteins are involved in environmental stress, PI-B05 may be important for understanding cold response in *Poncirus*. The full-length sequence of PI-B05 cDNA was obtained using 5' and 3' RACE. This cDNA was 1279 bp in length, consisting of a 146 bp 5'- untranslated region (UTR), a complete open reading frame (ORF) of 984 bp encoding a polypeptide of 328 amino acids, followed by a 3'- UTR of 147 bp (Figure 4-1).

Alignment of the protein encoded by this full-length cDNA sequence with other homologous proteins is shown in Figure 4-2. The multiple sequence alignment demonstrated that PI B05 shares significant sequence homology with previously characterized and putative AP2 domain containing proteins from different plants. The most conserved region of the protein was between amino acids 85-145 which contains the AP2 DNA binding domain. Sequence alignment of the AP2 domain from PI-B05 and the other plant proteins showed that the AP2 domain of PI-B05 was almost identical to the consensus sequence of AP2 domains (Figure 4-3), indicating that PI-B05 possibly contains a functional AP2 DNA binding domain.

The expression of PI-B05 in cold-acclimated and nonacclimated *Poncirus* and pummelo was studied by northern blot analysis to determine the expression pattern of this

A

B

Figure 4-1 Full-length sequence of *Poncirus* cDNA PI B05 A) The map of the full-length cDNA sequence showing open reading frame and the 5' and 3' untranslated regions B) The full-length cDNA sequence and the predicted translation of the PI-B05 open reading frame The amino acid sequences are shown on the top of the cDNA sequence

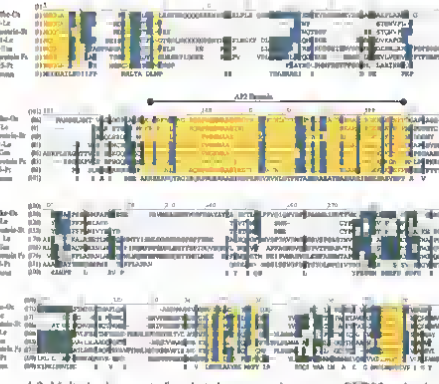


Figure 4-2 Multiple alignment of predicted amino acid sequences PI-B05 with other plant proteins in GenBank showing homology with PI-B05. The alignment was generated by the AlignX module of Vector NTI suite. GenBank accession numbers for ERF like protein from rice, (Os). ERF2 from tomato (Le), AP2 domain protein from potato (St), JERF1from tomato (Le), ERF from soy bean (Gm) and ERF protein *Fagus sylvatica* (Fs) are AAF05606, AAO34704, T07784, AAK95687, AAQ10777 and CAD21849 respectively. Conserved AP2 DNA- binding domain is indicated by the line above the sequence alignment.

A

B

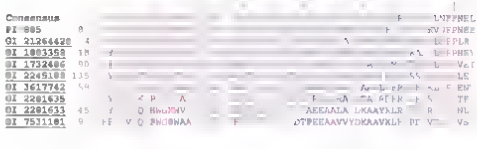


Figure 4-3 Analysis of amino acid sequences of PI-B05 cDNA A) The location of the conserved AP2 domain in the PI B05 amino acid sequence detected by Conserve Domain Search in the GenBank B) Alignment of the amino acid sequences of the conserved AP2 domain from *Poncirus* PI B05 with AP2 domain of proteins from other plants gi21264420_gi1903358 gi1732406_ gi2245108 gi3617742, gi2281635, gi2281633, gi7531181 are the GenBank id numbers for ERF1 from *Arabidopsis*, similar to *Nicotiana* EREBP 3 from *Arabidopsis*, S25 XP1 DNA binding protein from tobacco. EREBP-4 like protein from *Arabidopsis*, RAP2 6 *Arabidopsis*, AP2 domain containing protein RAP2.5 *Arabidopsis*, AP2 domain containing protein RAP2 4 *Arabidopsis*, PT16 from tomato

gene in two closely related species with cold hardy and cold-sensitive phenotypes Northern blot analysis revealed that in cold-hardy *Poncirus*, expression of this gene was induced at 1 h, reached peak levels at 2 d and remained at this high level following 4 d of cold acclimation, but decreased after 7 d of cold acclimation (Figure 4-4) However, only a small increase in expression was observed in cold-sensitive pummelo in response to cold starting at 1 d of cold acclimation remained relatively unchanged over the time points tested Based on northern blot analysis, the level of expression and induction of PI-B05 by cold was insignificant in pummelo compared to expression in *Poncirus* When expression of PI B05 was studied in dehydrated *Poncirus* and pummelo seedlings, no significant changes in expression of PI-B05 were observed, indicating that PI-B05 is only expressed in response to cold, but not dehydration

Cold-Induced PI-C10 cDNA from Poncirus Encodes a RAV-like Protein with two Different DNA-binding Domains

Reverse northern analysis of the partial cDNA sequence of PI-C10 (C10) showed an 11-fold induction and partial deduced amino acid sequences of this cDNA showed homology with the RAV-like protein from *Arabidopsis* Since RAV and RAV-like proteins contain two different DNA binding domains and it was shown that RAV1 was induced in response to cold in *Arabidopsis* PI-C10 may be involved in cold responsive gene regulation in *Poncirus* The full-length sequence of PI-C10 cDNA was obtained by 5' and 3' RACE using gene specific primers obtained from partial cDNA sequence The full-length PI-C10 cDNA was 1546 bp, consisting of a 159 bp 5' - UTR, a complete ORF of 1119 bp encoding a polypeptide of 373 amino acids, followed by a 3' UTR of 268 bp (Figure 4-5)

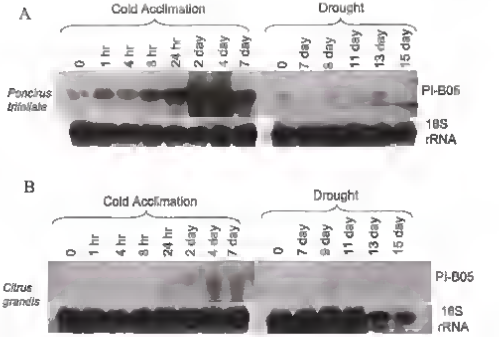


Figure 4-4 Northern blot analysis of expression of PI B05 cDNA in response to environmental stresses A) Expression of PI-B05 cDNA in response to cold acclimation and drought in *Poncirus* detected by antisense DIG-labeled riboprobe. B) Expression of PI B05 cDNA in response to cold acclimation and dehydration (drought) in Pummelo detected by antisense DIG labeled riboprobe. The type environmental stress treatment and the duration of the treatment are indicated on the top The expression of 18 S rRNA was used as a loading and transfer control and is shown below the expression of the specific gene.

Sequence analysis revealed that the protein sequence encoded by this cDNA showed sequence similarity with RAV1, RAV2 RAV2-like, and RAP2.8 proteins from *Arabidopsis* Multiple alignment of PI-C10 with these proteins presented in Figure 4-6 showed that this protein is highly homologous to this group of proteins encoding transcription factors, some of which are involved in cold-responsive gene expression PI-C10 showed high sequence identity in four different regions of RAV and RAV-like proteins. Sequence analysis showed that two of these regions were previously characterized AP2 and B3 DNA binding domains The AP2 and B3 domains are located towards the N terminous and C terminus of the protein, respectively (Figure 4-6) Multiple sequence alignment of AP2 and B3 domains of *Poncirus* PI-C10 revealed that both domains contain conserved amino acid sequences involved in DNA-binding (Figure 4-7) indicating that PI-C10 may have two functional DNA-binding domains.

To determine the expression pattern of this gene in two closely related species with cold-hardy and cold-sensitive phenotypes, the expression of PI-C10 in cold acclimated and nonacclimated *Poncirus* and pummelo was studied by northern blot analysis Expression of this gene was induced at 4 h and reached its highest level at 2 d, while it began declining starting at 4 d of cold acclimation in cold-hardy *Poncirus* (Figure 4-8). However no expression was detected in response to cold in cold-sensitive, pummelo (Figure 4-8) indicating that this gene is absent or its expression is too low to detect in pummelo by northern blot analysis When expression of PI-C10 was studied in dehydrated and nondehydrated *Poncirus* and pummelo plants, no expression of PI-C10 was detected in *Poncirus* or pummelo (Figure 4-8) indicating that PI-C10 is only expressed in response to cold, but not dehydration

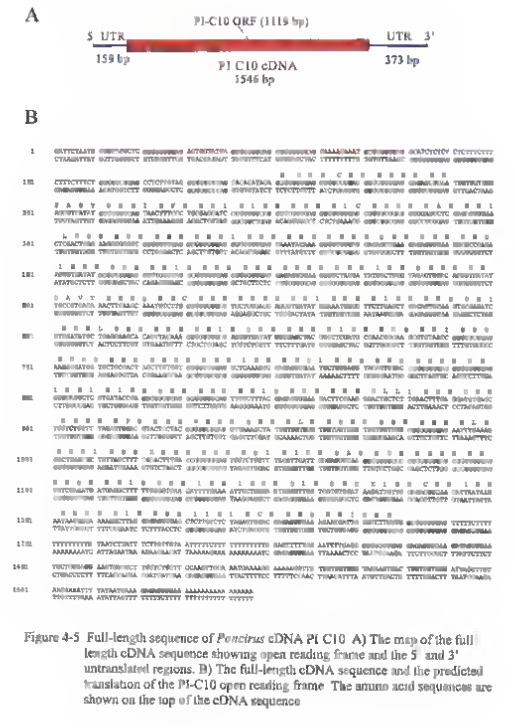


Figure 4-5 Full-length sequence of *Poncirus* cDNA PI C10 A) The map of the full length cDNA sequence showing open reading frame and the 5' and 3' untranslated regions. B) The full-length cDNA sequence and the predicted translation of the PI-C10 open reading frame The amino acid sequences are shown on the top of the cDNA sequence

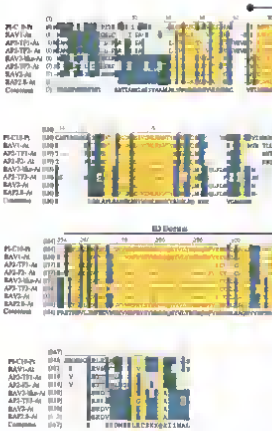


Figure 4-6 Multiple sequence alignment of predicted amino acid sequences of *Poncirus* PI C10 cDNA with proteins in GenBank showing the highest homology with PI C10. The alignment was generated using AlignX module of Vector NTI suite. The GenBank accession numbers for RAV1, AP2-TF1 (AP2 domain transcription factor), AP2 TF2 (putative AP2 domain transcription factor), RAV2-like (RAV2-like DNA binding protein) AP2 TF3 (putative AP2 domain transcription factor) RAV2 RAP2 8 from *Arabidopsis* are NP_172784, AAM65499 NP_189201, AAM63120 NP_173927, NP_564947, AAC49774, respectively. Conserved AP2 and B3 DNA-binding domains are indicated by the line above the sequence alignment.

Figure 4-7 Conserved DNA-binding domains of PI-C10. A) The location of the conserved AP2 and B3 domains in the PI-C10 amino acid sequence detected by Conserve Domain Search in the GenBank. B) Multiple sequence alignment of AP2 domain of PI C10 with AP2 domain of other plant proteins in GenBank. The GenBank identification numbers GI 21264420, GI 1903358, GI 3617742 GI 2811635, GI 2281647, GI 1171429, GI 2281641 and GI 7531181, represents ERF1 Similar to *Nicotiana* EREBP 3, RAP2.6, RAP2.11, RAP2 5, CKC RAP2 8 proteins from *Arabidopsis thaliana* and PTI6 tomato, respectively C) Multiple sequence alignment of B3 domain of PI-C10 with AP2 domain of other plant proteins in GenBank The GenBank identification numbers GI 12328560 GI 12328553, GI 6069650 GI 2281641, GI 3522951 GI 467820, GI 9758405 and GI 3695373 represent RAV2, putative RAV2-like, RAV2-like and P0514G12 9 proteins from *Oryza sativa* RAP2 8 RAV like B3 domain, putative RAV2, RAV2-like, and RAP2 8 like proteins from *Arabidopsis thaliana*, respectively

Since no expression of PI-C10 was detected in pummelo by northern blot analysis, RT-PCR was performed using primer sets specific to 5' and 3' regions of the full length cDNA to determine if this gene is absent or present in pummelo. RT-PCR amplification from total RNA of nonacclimated *Poncirus* and pummelo plants showed that the expression of PI-C10 was seen after 25 cycles of amplification in *Poncirus* and expression was only detected after 35 cycles of amplification in pummelo indicating that basal expression of this gene is very low in both plants (Figure 4-8 lanes 1-2 and 5-6). When the expression of this gene was studied in 2 d cold-acclimated plants, expression of PI-C10 was detected in both 25 and 35 cycles of PCR amplification in pummelo and *Poncirus* indicating that the PI-C10 isolated from *Poncirus* is also present in pummelo. However, the level of expression was significantly higher in *Poncirus* than pummelo at both 25 and 35 cycles of PCR amplification (Figure 4-8 lanes 3-4 and 7-8). These results suggested that although this gene is present in both species, its expression in response to cold acclimation of cold-hardy *Poncirus* and cold-sensitive pummelo is different.

PII-C02 cDNA from *Poncirus* Encodes a RING Zinc Finger Protein and is Induced in Response to Cold and Drought

Reverse northern analysis had revealed that PII-C02 (C78) showed a nine-fold induction upon cold acclimation. The partial deduced amino acid sequences of this cDNA showed homology with RING zinc finger proteins from other plants. At least one RING zinc finger protein from *Arabidopsis* is reported to be induced in response to cold. Thus, in determine whether the putative PII-C02 RING zinc finger protein might be involved in response to cold in *Poncirus*, the partial sequence was used for isolating full-length cDNA sequence of this gene. The full-length sequence of PII-C02 cDNA was

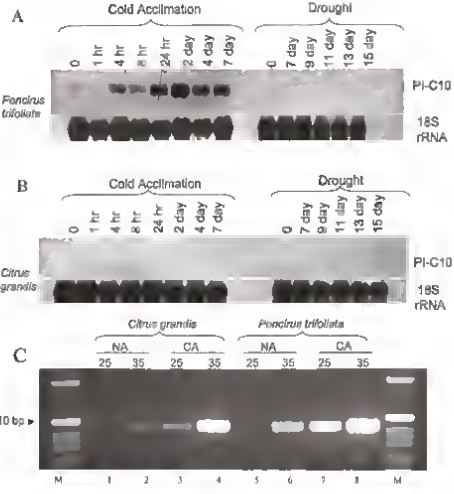


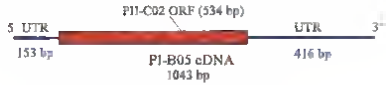
Figure 4-8 Expression of PI-C10 cDNA in response to environmental stresses. A) Northern blot analysis of expression of PI-C10 cDNA in response to cold acclimation and drought in Poncirus detected by antisense DIG-labeled riboprobe B) Northern blot analysis of expression of PI C10 cDNA in response to cold acclimation and drought in *Citrus grandis* (pummelo) detected by antisense DIG labeled riboprobe The type of environmental stress treatment and the duration of the treatment are indicated on the top The expression of the 18S rRNA was used as a loading and transfer control and is shown below the expression of the specific gene C) RT-PCR analysis of expression of PI C10 cDNA in non-acclimated (NA) and 2 day cold-acclimated (CA) Citrus grandis (pummelo) and *Poncirus trifoliata* The number of PCR cycles is indicated on the top and the size of the DNA marker (M) closest to the size of the amplified region of PI-C10 cDNA shown on the left side

obtained by 5' and 3' RACE using gene specific primers. This cDNA was 1043 bp, consisting of a 153 bp 5'- UTR, a complete ORF of 534 bp encoding a polypeptide of 178 amino acids, followed by a 3'- UTR of 416 bp (Figure 4-9)

Sequence analysis demonstrated that the deduced amino acid sequence of this full length cDNA showed homology with RING zinc finger proteins from *Thellungiella halophila* and *Arabidopsis*. Alignment of the protein encoded by this cDNA sequence with other RING zinc finger proteins is shown in Figure 4-10. The multiple sequence alignment demonstrated that PII-C02 shares significant sequence homology with previously characterized and/or putative RING zinc finger proteins from *Arabidopsis* and *Thellungiella halophile*. It contains a signature sequence motif for a RING zinc finger at the C terminus of the protein (Figure 4-10). The alignment of the PII-C02 RING zinc finger domain with proteins from plants and other organisms including human and fission yeast containing similar domains showed that the key amino acids in this domian were conserved in PII-C02, indicating that it might be a functional RING zinc finger protein (Figure 4-10). Since a histidine residue was found at the fifth coordination site of PII-C02, this protein is likely to belong to the RING-H2 subgroup.

The expression of PII-C02 was studied in response to cold and drought stresses to determine the expression pattern of this gene in two closely related species, *Poncirus* and pummelo. Northern blot analysis showed that expression of PII-CO2 increased inconsistently starting at 1 h of cold acclimation in *Poncirus*. Although the expression level was variable at different time points, expression reached its highest level between 24 h and 2 d of cold acclimation and decreased thereafter (Figure 4-11). Expression of PII-CO2 was at a basal level between 0 and 8 h, increased between 8 to 24 h of cold

A



B

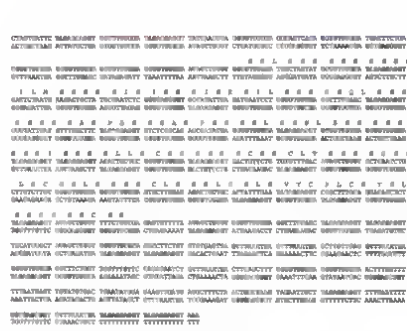


Figure 4-9 Full length sequence of *Fonctirus* cDNA PII C02 A) The map of the full-length cDNA sequence showing open reading frame and the 5' and 3' untranslated regions B) The full length cDNA sequence and the predicted translation of the PII-C02 open reading frame The amino acid sequences are shown on the top of the cDNA sequence

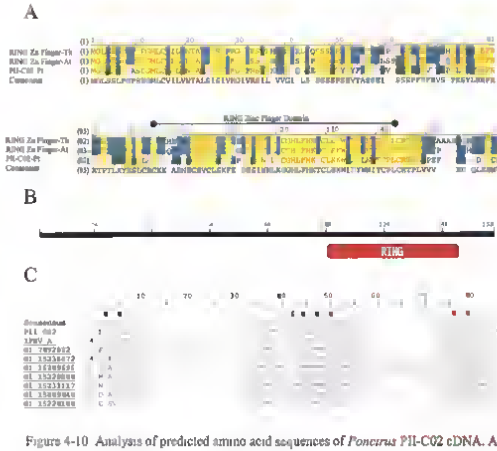


Figure 4-10 Analysis of predicted amino acid sequences of *Poncirus* PII-C02 cDNA. A) Multiple alignment of amino acid sequences PII-C02 with other plant proteins in GenBank showing homology with PII C02. The GenBank accession numbers for RING Zn FingerAAM19707 and AAG41449 represent putative RING zinc finger protein-like protein from *Thellungiella halophile* and *Arabidopsis thaliana*. B) The location of the conserved ring zinc finger domain in the PII-C02 amino acid sequence detected by Conserve Domain Search in the GenBank C) Multiple sequence alignment of the amino acid sequences of the conserved RING zinc finger domain from *Poncirus* PII C02 and other proteins containing similar domain. The conserved amino acid residues of the signature motif are indicated by # sign. The GenBank identification numbers GI 10120668, GI 7492802, GI 15238072, GI 16209696, GI 15229284, GI 15233117, GI 15809840 and GI 15228108 represent Chl-Ubch7 Ubiquitin Protein Ligases from human, PHD type RING zinc finger from fission yeast, RING-H2 zinc finger putative RING-H2 zinc finger F22D1 50 C3HC4-type RING zinc finger BRH1 RING zinc finger, putative RING-H2 zinc finger F17123 260 and C3HC4-type RING finger proteins from *Arabidopsis thaliana*

acclimation and dropped to the initial level after 2 d of cold acclimation in pummelo never reaching the levels attained in *Poncirus* (Figure 4-11). When the expression of PII-C02 was studied in response to drought, more significant changes in gene expression were observed both in *Poncirus* and pummelo. In *Poncirus* the expression of PII-C02 was induced at 7 d and reached its highest level at 9 d of dehydration, it started to decrease at 11 d and returned the initial level at 13 d. On the other hand, in pummelo, the expression of PII-C02 was induced at 7 d and reached its highest level between 9 and 11 d and started to decrease after that time. The apparent high level of expression observed at 15 d of dehydration may be due to a higher amount of RNA in that sample since the amount of 18S ribosomal RNA is also higher in that sample. These results showed that PII-C02 encoding a putative RING zinc finger protein is induced by both cold and drought, however, the level of induction in response to drought is much higher than the cold-responsive expression.

Discussion

The gene expression studies in cold acclimated and nonacclimated *Arabidopsis* and other plants have shown that expression of several hundred genes is changed in response to cold. Genes induced in response to cold are involved in a variety of cellular functions including transcriptional regulation. A family of AP2 domain containing transcription factors, the CBF/DREB family, was isolated from *Arabidopsis* and shown to regulate cold-responsive gene expression (Stockinger et al. 1997, Liu et al. 1998, Gilmour et al. 1998). Homologs of CBF/DREB genes were identified and isolated from several groups of plants, indicating that the CBF/DREB pathway is conserved at least among some quite disparate plants (Jaglo et al. 2001). However, the presence of the CBF/DREB pathway in other plants including *Poncirus* is still unknown. In addition to

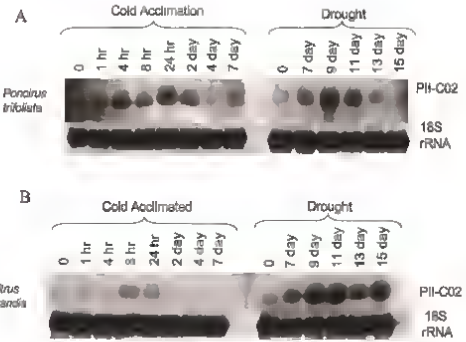


Figure 4-11 Northern blot analysis of expression of PII-C02 cDNA in response to environmental stresses A) Expression of PII-C02 cDNA in response to cold acclimation and drought in *Poncirus* detected by antisense DIG labeled riboprobe. B) Expression of PII-C02 cDNA in response to cold acclimation and drought in pummelo detected by antisense DIG-labeled riboprobe. The type environmental stress treatment and the duration of the treatment are indicated on the top. The expression of 18 S rRNA was used as a loading and transfer control is shown below the expression of the specific gene

the CBF/DREBs, expression of a number of other transcription factors with conserved DNA binding domain(s) such as bZIP bHLH, zinc finger, MYB home box, MADS box, as well as AP2/EKF, AP2/B3 and WRKY proteins were induced in response to cold (Kagaya et al 1999, Chen et al 2002, Seki et al 2001, 2002, Fowler and Thomashow 2002, Sakuma et al 2002) Expression of some of these transcription factors is controlled by CBF/DREB however, the expression of other transcription factors is independent of CBF/DREB, indicating that pathways other than CBF/DREB are activated in response to cold (Fowler and Thomashow 2002)

Three partial cDNAs, PI-B05, PI-C10 and PII-C02, showing homology to transcription factors were selected from a cDNA library prepared from 2 d cold-acclimated *Puccinellia tryfoliata*. The full-length sequences of these cDNAs were obtained by 3' and 5' RACE. Analyses of nucleotide and deduced amino acid sequences of genes encoded by these cDNAs revealed that PI-B05, PI-C10 and PII-C02 encode proteins containing an AP2 DNA binding domain, AP2 and B3 DNA-binding domains, and a RING zinc finger domain, respectively

Sequence analysis of PI-B05 demonstrated that it contains an AP2 DNA-binding domain that is similar to a DNA binding domain of previously characterized and putative proteins from *Arabidopsis* and other plants, including potato, tomato and rice. Since this protein contains only one AP2 domain it can be classified along with single AP2/ERF containing proteins, including ERF, TINY, DREB1/CBF, DREB2, Pti5, and ABI4 (Riechmann and Meyerowitz 1998, Riechmann et al 2000, Sakuma et al 2002). Closer analysis of the conserved sequences in the AP2 domain revealed that PI-B05 is most similar to the ERF group of proteins because unlike the DREB1/CBF group, this

group contains alanine and aspartic acid at position 14 and 19, respectively. It was shown that these two amino acids in the AP2 domain determine DNA-binding specificity of proteins (Sakuma et al. 2002). The DREB1/CBF group contains valine and glutamic acid at position 14 and 19, respectively and binds specifically to the CRT/DREB binding element A/GCCGAC. On the other hand, ERF-like proteins bind to the GCC box containing the core AGCCGCC sequence demonstrating that a single base change determines the specificity of these two groups of AP2/ERF proteins (Sakuma et al. 2002). The GCC-box binding specificity of the ERF proteins ERF1 from *Arabidopsis* and ERF2 from tomato, which contain DNA-binding domains highly similar to PI-B05, have been demonstrated (Allen et al. 1998, Tournier et al. 2003). The 3-dimensional structural analysis of the GCC-box binding domain of ERF1 from *Arabidopsis* with the GCC box sequence revealed that it contains a three-stranded anti-parallel beta-sheet and an alpha-helix packed approximately parallal to the beta-sheet. The domain recognizes the target sequence through a β-sheet structure (Allen et al. 1998). Since PI-B05 contains an AP2 domain very similar to this protein, it is likely that it has the same domain structure and binds to a GCC-box. The expression of ERF genes was differentially induced by ethylene and by abiotic stress conditions, including cold, high salinity, or drought, through ETHYLENE-INSENSITIVE2 (EIN2)-dependent or EIN2-independent pathways (Fujimoto et al. 2000). A number of AP2/ERF proteins are also involved in expression of pathogenesis related genes. Thus, a group of AP2/ERF proteins have roles in biotic and abiotic stress-induced gene regulation. Expression analysis of PI-B05 in response to cold and drought in *Poncirus* and pummelo demonstrated that this gene is only induced by cold in *Poncirus*, strongly suggesting that it regulates cold-responsive gene expression in

Poncirus under cold stress. Since the AP2 domain of this protein may have binding specificity to a GCC-box sequence- genes regulated by this protein should contain such a GCC box regulatory sequence

Sequence analysis of PI-C10 showed that this protein contains two different DNA binding domains, AP2 and B3, primarily found in the plant transcription factors RAV1 and RAV2 from *Arabidopsis* (Kagaya et al 1999) Searches in the sequence database identified a number of other RAV-like proteins from *Arabidopsis* and other plants. However, these genes have not been characterized Analysis of the DNA-binding activity of RAV1 protein by deletion and electrophoretic mobility shift assays demonstrated that the AP2 and B3 domains independently bind to CAACA and CACTCG sequence motif, respectively The presence of two DNA-binding domains provides higher binding affinity and specificity to the RAV1 protein (Kagaya et al 1999). Since the amino acid sequence of AP2 and B3 domain of *Poncirus* PI-C10 is almost identical to the respective domains from RAV1 and RAV2 from *Arabidopsis*, it is likely that they will have the same DNA-binding activity and will bind to CAACA and CACTCG sequence motifs. Currently, the target genes of RAV proteins are not known, however, recent microarray studies show that at least RAV1 from *Arabidopsis* is induced in response to cold acclimation (Fowler and Thomashow 2002), indicating that it is involved in regulation of cold responsive gene expression Expression analysis of RAV like PI-C10 by northern blot hybridization in this study demonstrated that it is induced only in response to cold in cold hardy *Poncirus* but not to drought and no expression was detected in pummelo in response to both cold and drought This result confirms the suggestion that the RAV proteins are involved in cold-regulated gene expression and PI-C10 most likely regulates gene

expression specifically under cold stress in *Poncirus*. Further expression analysis of PI-C10 by RT-PCR showed that this gene is also present in cold-sensitive pummelo, however, the level of expression is significantly higher in cold-hardy *Poncirus*. The differences observed in expression of this gene in pummelo between northern blot and RT-PCR analyses may be due to the sensitivity of the two detection methods or sequence variations within this gene in pummelo and *Poncirus*. Since hybridization stringency used in northern blot analysis allows 20% sequence variations and two different regions of PI-C10 were amplified by PCR using two different sets of primer designed from non-conserved regions of this gene, it is unlikely the differences are due to sequence variations. Thus, it is likely that the differences in expression of PI-C10 in pummelo are due to the increased sensitivity provided by RT-PCR. Since the expression signal is amplified exponentionally by PCR, RT-PCR is much more sensitive than the northern blot analysis and it can detect lower amounts of RNA signal.

Database searches with the predicted amino acid sequence of PI-C02 from *Poncirus* identified two *Arabidopsis* proteins showing high sequence identity. It also showed homology with a number of other proteins from plants and other organisms. The most similar regions of these proteins contained a RING zinc finger motif found near the C terminus suggesting that PI-C02 is a RING zinc finger protein. The alignment of the amino acid sequences of the RING zinc finger domains shows that PI-C02 contains key conserved amino acids in the correct positions. It also revealed that PI-C02 has a histidine residue at the fifth coordination site, suggesting that it is a member of the H2 subclasses of RING zinc finger proteins which is mostly found in plants.

RING zinc finger proteins are thought to function as transcription factors because of the presence of zinc finger motifs in their sequences. Although the exact function of most RING zinc finger proteins is still unknown, recent studies with some mammalian RING zinc finger proteins demonstrated that they are involved in specific ubiquitination of proteins and suggested that these proteins act as ubiquitin protein ligases (Freemont 2000 Joazeiro and Weissman 2000) Since this pathway is conserved in all organisms and regulates the specific degradation of a large number of proteins involved in diverse cellular processes ranging from cell cycle regulation to stress responses (Hershko and Ciechanover 1998), RING zinc finger proteins may have important roles in these processes. The *Arabidopsis* genome contains a number of RING finger proteins (Jensen et al 1998) Although a majority of these proteins have not been characterized and no functions have been assigned to them, more and more studies are being conducted to elucidate the function of some of these proteins. Recent characterization of a small number of plant RING zinc finger proteins revealed that they are involved in key biological processes of plants including photomorphogenesis (Torii et al 1999), seed development (Molnar et al 2002, Lechner et al 2002), pathogen defense (Takai et al 2002, Guo et al 2003) and cold-responsive gene expression (Lee et al 2001) Although ubiquitin ligase activity has not been shown for any of these proteins, current data suggests that these proteins function through protein-protein interactions and the RING zinc finger domain is essential for their activity (Torii et al 1999, Molnar et al 2002, Lechner et al 2002 Takai et al 2002, Guo et al 2003, Lee et al 2001) HOS is a RING zinc finger containing protein that functions as a repressor of cold-induced gene expression through CBF/DREB and possibly other pathways in *Arabidopsis* (Lee et al.

2001). More recently microarray analysis of a large portion of the *Arabidopsis* genome revealed that expression of at least one RING zinc finger protein was induced in response to cold (Fowler and Thomashow 2002), indicating that RING finger proteins may also be involved in regulation of cold or other environmental stress-induced gene expression. In this study, expression of a RING zinc finger domain containing PH-C02 was induced in response to both cold and drought in *Poncirus* and pummelo. Since the expression level of PH-C02 was much higher in dehydrated plants than the cold-acclimated plants, PH-C02 regulation of gene expression may be more important in Poncirus and pummelo under drought stress than cold stress.

Three genes identified and characterized in this study showed cold-responsive induction and differential expression in cold-hardy *Poncirus* and cold-sensitive pummelo. Since the expression of all three genes is significantly higher in cold-acclimated Poncirus than pummelo, they may contribute to cold hardiness in *Poncirus*. Since two of these genes encode proteins with highly conserved DNA-binding domain(s), they are likely to be involved in transcriptional regulation of other cold-induced genes in *Poncirus*. Therefore, not only these genes, but downstream genes potentially activated by these proteins could contribute to cold hardiness in *Poncirus*. Although this study clearly establishes the cold regulated nature of these genes, further studies are needed for determination of their actual functions and involvement of these genes in cold hardiness in *Poncirus* and their potential use for improving cold tolerance in *Citrus*.

CHAPTER 5
SUB-CELLULAR LOCALIZATION OF COLD REGULATED GENES COR11 AND COR19

Introduction

Dehydrins are a family of proteins that are commonly induced in response to environmental stress such as dehydration, low temperature, salinity, as well as in response to ABA treatment and during embryo development (Close 1996). They are also referred to as group 2 late embryogenesis abundant (LEA) proteins since they were first identified during embryo development in cotton (Baker et al. 1988). Dehydrins are evolutionarily conserved among photosynthetic organisms as well as in yeast. They have been isolated from gymnosperms and angiosperms and the existence of dehydrins has also been detected in algae, cyanobacteria and yeast by immunological analysis using antibodies specific to dehydrins (Close 1997, Mtwisha et al. 1998). The common features of this family of proteins include high hydrophobicity, thermostability and presence of multiple tandem repeats containing highly conserved consensus sequences.

Dehydrins are characterized by conserved Y-, S-, K- and Φ segments found in their amino acid sequences (Close 1996). The numbers of these various segments can be used for determining sub-classes of dehydrins and for comparison between alleles of individual dehydrin genes (Campbell and Close 1997, Choi et al. 1999). The Y-segment is composed of V/TDEYGNP and one to three copies of it is usually found close to the N-terminus of many dehydrin proteins. The S-segment contains five to seven serine residues followed by three acidic amino acids and is located downstream of the Y

segment of some dehydrins (Close 1996) The S-segment is phosphorylated and may be involved in nuclear localization of maize dehydrin RAB17 (Goday et al 1994) and tomato dehydrin TAS14 (Godoy et al 1994). The K segment consists of the 15-amino acid lysine rich sequence EKKGIMDKIKEKLPG and is found near the C-terminus of all dehydrins, repeated one to 11 times The K segments are proposed to form class A amphipathic α helices (Dure 1993, Close 1996) which may act as an interphase between hydrophobic surfaces of membrane phospholipids and the cytosol to protect membrane function. In addition they may interact with partially denatured proteins to prevent aggregation of proteins and to protect functions of proteins under environmental stress (Close 1996, 1997). The majority of dehydrins contain a Φ-segment which is rich in glycine and polar amino acids and is tandemly repeated between K segments It has been proposed that the highly polar Φ-segment of dehydrins interacts with polar groups on macromolecules in the cytoplasm and nucleus to prevent coagulation of macromolecules (Close 1997)

Since dehydrins are induced in response to environmental stress, it was suggested that they have a role in dehydration induced stress tolerance in plants Dehydrins protect integrity of cells by stabilizing membranes and proteins through detergent or chaperone like activities (Close 1996 1997, Ismail et al. 1999) Increased chilling tolerance in cowpea is not correlated with reduced electrolyte leakage, indicating that the 35 kDa dehydrin might not be protecting the plasma membrane, but that it reduces dehydration induced damage by interacting with internal membranes of the cells instead (Ismaïl et al 1999) It was also proposed that COR15am dehydrins from *Arabidopsis* interact with plant membranes and protect them from freezing induced lamellar-to-hexagonal phase

transitions (Thomashow 1999). Recently it was suggested that the COR19 from *Citrus unshiu* enhanced cold tolerance in transgenic tobacco by inhibition of lipid peroxidation (Hara et al. 2003).

Dehydrins display diverse tissue specific and sub-cellular localization patterns. Immunolocalization and sub-cellular fractionation studies with a number of dehyrins demonstrated that they are mostly localized in the nucleus and cytoplasm (Close 1996). However, some dehydrins have been found to be associated with plasma membranes (Danyluk et al. 1998) chloroplasts (Wisniewski et al. 1999) and mitochondria (Borovskii et al. 2000, 2002, Hara et al. 2003). Many dehydrins localized in the nucleus contain putative bipartite nuclear localization signals (NLS) (Monroy et al. 1993, Goday et al. 1994, Godoy et al. 1994, Cai et al. 1995, Houde et al. 1995). The nuclear localization of some dehydrins is tissue specific (Asghar et al. 1994), some such as RAB17 from maize and TAS14 from tomato require post-translational modification (Goday et al. 1994, Godoy et al. 1994) and others can only be localized in the nucleus under certain environmental stress such as cold (Rinne et al. 1999).

Two cold regulated genes COR11 and COR19 were isolated from seven-day cold acclimated *Poncirus trifoliata* which is a cold-hardy citrus relative (Cai et al. 1995). Expression of both genes was induced in response to cold, but repressed in response to drought and flooding. Both genes contained repeats of conserved lysine-rich K-segment within the protein and S-segment similar to other dehydrins near the C terminus. In addition, three Q-cluster regions containing a conserved H2Q3 5YR sequence motif and a putative bipartite NLS were also found in both COR11 and COR1 (Figure 5-1) (Cai et al. 1995). To determine if the NLS found in the cold regulated genes from *Poncirus* are

functional, sub-cellular localization of the products of these genes was analyzed in onion epidermal cells

Materials and Methods

Amplification COR Genes and Cloning of corrgus Fusion Constructs

COR11 and COR19 genes were amplified by RT-PCR using specific primers First, cDNA was synthesized from total RNA isolated from two-day cold-acclimated *Poncirus trifoliata* using Superscript II reverse transcriptase and oligo-dT primer (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Then, complete open reading frames of COR11 and COR19 were amplified from the cDNA by PCR with *Pfu* DNA polymerase using primers specific to the 3 and 5' ends of the individual genes. *Nco*I and *Bgl*II sites were incorporated at the 5' and 3' ends of both genes, respectively and the stop codons of both genes were deleted during PCR amplification Since the putative NLS signal in the C terminal half of the COR11 and COR19 genes was almost identical, the N-terminal half of COR11 was deleted by PCR amplification of the C-terminal half of this gene by PCR with *Pfu* Polymerase using an internal primer with an *Nco*I site and the same 3'terminal primer with a *Bgl*II site and without the stop codon The PCR products were purified using the Qiquick PCR Purification Kit (QIAGEN, Hilden, Germany) digested with *Nco*I and *Bgl*II restriction enzymes (Invitrogen Carlsbad, CA) and cloned into pCAMBIA 1301 binary vector cut with the same restriction enzymes to fuse the COR sequences to a β-glucuronidase (GUS) reporter gene in frame (Figure 5-1) The resulting plasmids were designated as pCAMBIA 1301 - COR11 COR19 and COR11C

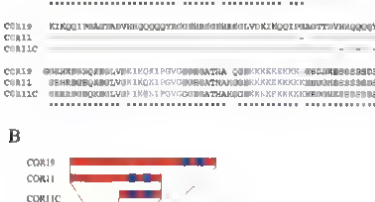


Figure 5-1 Sequence alignment and cloning of COR genes for sub-cellular localization study A) Amino acid sequence alignment of full length COR11, COR19 and C terminal half of COR11 (COR11C) The asterisks and dashes indicate identical or absent amino acid residues respectively The sequences of bipartite NLS signals are shown in blue B) Cloning of COR11, COR19 and COR11C into the pCAMBIA 1301 binary vector to generate cor::gus fusion constructs The red boxes indicate the genes or portions of genes cloned into the binary vector and the small blue boxes show the approximate locations of bipartite NLS in individual genes. The components of binary vector are shown in the vector map

Particle Bombardment of Onion Epidermal Cells

The plasmids containing cor- gus fusion constructs pCAMBIA 1301-COR11 COR19 and COR11C as well as control plasmid pCAMBIA 1301 were purified using Qiagene Miniprep Kit (QIAGEN, Hilden, Germany) and concentrations of DNAs were determined spectrophotometrically. Tungsten particles (30 mg) were incubated in 70% ethanol for 15 min, washed three times with sterile water, and resuspended in 50% glycerol to obtain the concentration of 60 mg/ml. A 50 µl tungsten suspension was mixed with 5 µg DNA from each construct, 16 mM spermidine, and 10 mM $CaCl_2$, vortexed for 3 min, and incubated for 1 min at room temperature. After two washes with 70% ethanol, the tungsten particles coated with DNA were resuspended in 48 µl of 100% ethanol. An aliquot (8 µl) of tungsten particles was placed onto a microcarrier for particle bombardment. Inner epidermal layers of onion (*Allium cepa*) were peeled and placed on petri dishes containing Murashige-Skoog media (Sigma, St. Louis, MO) pH 5.7 [4.3 g MS salts (Invitrogen, Carlsbad, CA), 1 mg thiamine, 10 mg myo-inositol, 180 mg KH_2PO_4, 30 g sucrose, and 6 g agar] with 2.5 mg/l amphotericin B (Sigma). Epidermal cell layers of onions were bombarded at 1100 psi using the Biolistic PDS-1000/He System (Bio-Rad, Richmond, CA). Bombarded onion samples were maintained at 28 °C for 24-48 h and analyzed for the expression of GUS or they were first maintained at 28 °C for 24-48 h then transferred to 4 °C for up to five days before analysis for GUS expression.

Subcellular Localization of COR Gene Products in Onion Epidermal Cells

Bombarded onion epidermal cells were removed from media and fixed in 50 mM phosphate buffer pH 7.0 solution containing 1.5% formaldehyde, 0.05% TritonX-100 for

30 min at room temperature. After washing four times with 50 mM phosphate buffer for 15 min each fixed epidermal cells were transferred to (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-gluc) solution containing 0.5 mM X-gluc, 50 mM phosphate buffer pH 7.0, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide, and 0.1% TritonX-100 and incubated at 37 °C for 6-16 hours. After GUS staining, samples were transferred to water and analyzed for expression and localization of GUS in the epidermal cells by light microscopy (Zeiss). Glass microscope slides were prepared from GUS stained samples and a 4',6-Diamidino-2-phenylindole (DAPI) solution (20 pg/mL DAPI, 0.1X PBS, 10 mM Na azide, 90% glycerol) applied onto the slides to localize the nuclei as previously described by McLean et al. (1990). The slides were examined under light microscopy to observe GUS expression and localization of the nuclei stained with DAPI and photographed using a camera attached to the microscope.

Results

To determine if the previously reported bipartite nuclear localization signals (NLS) of two cor genes of Poncirus, COR11 and COR19, are functional, these genes were fused to a GUS reporter gene in the pCAMBIA 1301 binary vector (Figure 5-1). Epidermal cells were bombarded with control plasmid 1301 and plasmid containing the fusion constructs (1301-COR11, COR19, and COR11C). Histochemical GUS assays showed that all constructs resulted in GUS expression in the onion epidermal cells, indicating that all constructs were functional and the particle bombardment was successful. Analysis of GUS expression and its distribution in the cells with microscopy demonstrated that in onion epidermal cells bombarded with control plasmid pCAMBIA 1301 blue GUS staining was evenly distributed in the cytoplasm, indicating that the GUS localized in the cytoplasm and did not reach the nucleus by diffusion (Figure 5-2). In contrast to1301

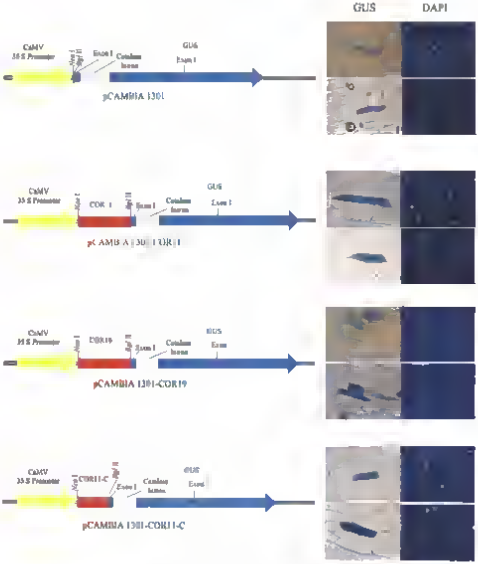


Figure 5-2 Sub-cellular localization of COR genes in onion epidermal cells The maps of partial T-DNA regions of the control plasmid and plasmids containing COR gene sequences fused to the GUS reporter gene are shown on the left. Pictures showing the expression and distribution of GUS in onion epidermal cells bombarded with control and nuclear localization constructs are presented on the right side of the individual constructs. Pictures on the left panel present the histochemical GUS staining showing the expression and distribution of GUS in the onion epidermal cells bombarded with specific constructs. Pictures on the right panel present the DAPI staining showing the location of the nucleus in the onion epidermal cells

the expression of GUS was concentrated mainly in the nucleus, with some GUS expression observed in the cytoplasm of the epidermal cells of onion bombarded with COR11 and COR19 fusion constructs. The results demonstrated that the COR genes are able to localize GUS in the nucleus of onion epidermal cells indicating that the previously proposed nuclear localization signals of these two genes are functional. When the GUS staining was done for a short period of time (6 h) and/or the expression of GUS in the individuals cells was low, GUS expression was first seen in the nucleus and as the expression increased, the GUS staining started to appear in the cytoplasm (data not shown), indicating that the nucleus is the primary target for these proteins. To determine if temperature has any effect on nuclear localization of these gene products, after initial incubation at 28°C, the bombarded epidermal cells were transferred to 4°C. No difference in GUS staining was observed between the samples kept at 28°C and the ones transferred to 4°C, indicating that temperature has no effect on nuclear localization of these proteins in onion epidermal cells.

Since the nuclear localization signals of COR11and COR19 were found close to the C-terminus of the proteins, the N- terminal half of COR11 was deleted and only the C-terminal half of the protein was fused to GUS gene in the binary vector to generate pCAMBIA 1301-COR11C (Figure 5-1). When the onion epidermal cells were bombarded with this construct, a high level of GUS expression was observed in nuclei which were identified by DAPI staining and some GUS expression was also found in the cytoplasm, showing that the C-terminal half of the COR11 is sufficient for the nuclear localization of the fusion GUS reporter gene (Figure 5-2). As the other constructs, temperature had no effect on nuclear localization of this construct.

Discussion

Two cold regulated genes, COR11 and COR19, showing homology to dehydrins were previously isolated from *Poncirus trifoliata*. These genes not only contained conserved signature domains of K- and S-segments but also contain sequences similar to previously characterized bipartite NLS (Cai et al. 1995). Since it has been proposed that dehydrins are involved in protection of membranes and proteins of cells under environmental stress conditions (Close 1996, 1997, Ismail et al. 1999), the sub-cellular location of these proteins are important for understanding their function in the cell. Therefore, tissue distribution and sub-cellular localization of dehydrins have been explored extensively to assign function(s) for specific dehydrins isolated from different plants. Immunolocalization and sub-cellular fractionation assays revealed that the majority of dehydrins are localized both in the nucleus and cytoplasm of different plant cells (Monroy et al. 1993, Goday et al. 1994, Godoy et al. 1994, Houde et al. 1995, Close 1996, 1997).

Sub-cellular localization of COR11 and COR19 in onion epidermal cells showed that these proteins are localized in the cytoplasm and nucleus indicating that in addition to sequence homology, *Poncirus* dehydrins may be functionally similar to previously characterized dehydrins from other plants which are localized in the nucleus and cytoplasm (Monroy et al. 1993, Goday et al. 1994, Godoy et al. 1994, Houde et al. 1995, Close 1996). Localization of these proteins in the nucleus and cytoplasm suggests that they may be involved in the protection of membranes and macromolecules in the nucleus and cytoplasm as was proposed for other dehydrins (Close 1997). The expression level of the GUS reporter gene was consistently and significantly higher in the nucleus than the cytoplasm of onion epidermal cells bombarded with all three constructs in repeated

experiments. In addition, when GUS expression was analyzed early following bombardment (6-hr) and/or the level of GUS expression was low in individual cells, the proteins were primarily localized in the nucleus. These results suggested that although COR11 and COR19 were found both in the nucleus and cytoplasm, the primary target of these proteins is the nucleus. Accumulation of COR11 and COR19 primarily in the nucleus implies that they may be involved in protection of macromolecules in the nucleus such as regulatory proteins and/or transcriptional machinery under cold stress. A similar function was also suggested for a wheat dehydrin, WCS120, which is localized in the nucleus and cytoplasm of wheat crown tissue (Houde et al. 1995).

Some dehydrins are localized in the nucleus after posttranslational modification of the proteins through phosphorylation of S residues in the S-segment (Goday et al. 1994, Goday et al. 1994) and others require certain environmental stress such as cold for nuclear localization (Rinne et al. 1999). Since maintaining bombarded cells at 28 and 4 °C did not have any effect on the distribution of the proteins in the cell, unlike the RAB16 dehydrin from birch, *(Betula pubescens)* (Rinne et al. 1999) neither of the *Poncirus* dehydrins require change in temperature for their localization in the nucleus. The phosphorylation status of the S-segment of COR11 and COR19 and its effect on the nuclear localization was not studied and is still unknown.

It has been reported that dehydrins from some plants such as RAB21 from rice and CAP85 from spinach were principally or exclusively localized in the cytoplasm (Mundy and Chua 1988, Neven et al. 1993). More recently it has been shown that dehydrins from some plants localize primarily in chloroplasts (Wisniewski et al. 1999) or plasma membranes (Danyluk et al. 1998). In this study, no specific localization of COR

genes in cell walls or plasma membranes of onion epidermal cells was observed. Other dehydrins, including the COR19 from *Citrus unshiu* (CuCOR19) which is a homologue of COR19 from *Poncirus* used in this study, were mainly localized in the mitochondria (Borovsky et al. 2000, 2002 Hara et al 2003) Sub-cellular fractionation of transgenic tobacco expressing CuCOR19 showed that although the main location of the CuCOR19 was the mitochondria some of the protein was found in the nucleus (Hara et al 2003) indicating that even very similar dehydrins from closely related species may show different sub-cellular localization The variation in the localization patterns of these two proteins may be due to the differences in the method or the type of plant cell used for sub-cellular localization studies. Constitutive expression of CuCOR19 in tobacco decreased electrolyte leakage by reducing lipid peroxidation and the CuCOR19 protein expressed in bacteria prevented peroxidation of soybean liposomes in vitro Based on these results it was suggested that this dehydrin increased cold tolerance by protecting the integrity of membranes of the cell by decreasing peroxidation in tobacco This study was focused on sub-cellular localization of two dehydrins from *Poncirus* and provided experimental evidence for localization of these proteins in the nucleus and cytoplasm. Understanding of the role of *Poncirus* COR11 and COR19 genes in cold tolerance and their possible mechanism of action will require further experiments in *Poncirus* or other plants.

CHAPTER 6
SUMMARY AND CONCLUSIONS

Citrus is one of the most important fruit crops in the world with an annual production exceeding one hundred million metric tons. Production of citrus is mostly limited by low temperatures outside the tropical and subtropical regions. Most commercial citrus types are cold-sensitive and subjected to low temperatures and freezes in the subtropical regions. Occasional freezes cause significant damage and economic losses in some citrus growing regions, including Florida. Therefore, improving cold tolerance of commercial varieties is an important goal for the citrus industry. *Poncirus trifoliata* is a cross compatible *Citrus* relative and unlike *Citrus*, it can tolerate temperatures as low as -26 °C when it is cold-acclimated. Thus, it serves as good genetic resource for improving cold tolerance in Citrus. Because of this characteristic, it has been used in breeding programs; however, mechanisms of cold response in *Poncirus* have not been explored at the molecular level.

To identify genes induced during cold acclimation, reverse and forward subtracted cDNA libraries were prepared using cold-acclimated and nonacclimated *Poncirus* seedlings. A total of 192 randomly picked colonies, 136 from forward and 56 from reverse subtracted libraries were sequenced and they showed that a number of cDNA clones had homology to previously characterized cold response genes in other plants. Expression studies with reverse northern blot analysis demonstrated that expression of 97 cDNAs was changed two to 49 fold, indicating that they are cold-regulated. Among them 96 were induced and only one was repressed by cold. Cold-

Induced genes identified in this study have homology to different groups of genes, including transcription factors and DNA binding proteins, heat shock proteins LEAs, and some metabolic genes. Differential expression of 17 selected cDNAs in response to cold was confirmed by northern blot analysis using gene specific probes in cold-acclimated and nonacclimated *Poncirus* plants

Gene expression studies in other plants revealed that multiple pathways are involved in regulation of gene expression during cold acclimation Expression of cold-regulated genes is controlled by a number of transcription factors, some of which have already been characterized A number of cDNAs from our cold-induced subtractive cDNA library show homology to previously characterized or putative transcription factors Three of these cDNAs PI-B05 PI-C10 and PII-C02 were selected for further characterization. The full-length sequences of these cDNAs were obtained by 3' and 5' RACE using gene specific primers obtained from partial cDNA sequences.

Sequence analysis of the full-length cDNA of PI-B05 encodes a protein containing an AP2 DNA binding domain and showed homology with previously characterized AP2 domain transcription factors from *Arabidopsis* and other plants. Northern blot analysis revealed that expression of this gene was induced in response to cold acclimation in *Poncirus*, but not in pummelo No changes in expression were detected in response to drought either in *Poncirus* or pummelo, indicating that this gene is induced only by cold in *Poncirus*

The full-length cDNA of PI-C10 codes for a RAV2-like protein with two different, AP2 and B3, DNA binding domains It showed homology to previously characterized RAV1 and RAV2-like proteins from other plants. When the expression of

PI-C10 was studied in response to cold and drought in *Poncirus* and pummelo, expression of PI-C10 was increased only in response to cold in *Poncirus*. No expression was detected in response to drought in Poncirus or in response to cold and drought in pummelo suggesting that PI-C10 is a cold-regulated gene in *Poncirus*

Sequence analysis revealed that the PII-C02 cDNA encodes a RING zinc finger protein that showed homology with two RING zinc finger proteins from *Arabidopsis*, one of which is induced by cold. Northern blot analysis showed that the expression of PII-C02 was induced in response to cold and drought in both *Poncirus* and pummelo. The increase in gene expression was more significant in response to drought in both plants

Sub-cellular localization of COR11 and COR19 in onion epidermal cells with GUS fusion constructs showed that these proteins are localized in the nucleus and cytoplasm, but accumulated predominantly in the nucleus. Deletion of the N-terminal half of COR11 demonstrated that C-terminal half conserved in both genes was sufficient for nuclear localization.

This study reports the first comprehensive analysis of gene expression during cold acclimation and identification of over 90 cold-regulated genes in cold-hardy *Poncirus*. The results of expression analysis of cold-regulated genes by various methods lead to following conclusions: (1) Identification of many genes regulated by cold confirms the quantitative nature of the cold hardiness trait in *Poncirus*; (2) Cold-regulated genes identified in *Poncirus* are involved in a variety of cellular functions indicating that cold acclimation induces many biochemical and physiological changes in *Poncirus* as in other plants. (3) Most of the cold regulated genes identified in *Poncirus* are similar to cold regulated genes in *Arabidopsis* and other plants suggesting the presence of similar cold

responsive pathways are activated during cold acclimation (4) Some of cold-regulated genes are induced in cold-hardy *Poncirus*, but not in cold-sensitive pummelo indicating that differential expression of these genes and others may be responsible for cold hardiness in *Poncirus*

The purpose of this project was to study changes in gene expression during cold-acclimation in cold-hardy citrus relative *Poncirus* Using subtractive hybridization, reverse northern, and northern blot analysis more than 90 genes were identified Although this may not be the complete list of genes regulated by cold in *Poncirus*, this study provides the first comprehensive information about changes in gene expression during cold acclimation The genes identified in this study especially the ones encoding regulatory proteins, should be further characterized to elucidate their functions and involvement in cold hardiness. Since two of these genes encode proteins with conserved DNA-binding domain(s) and show homology with previously characterized transcription factors, DNA binding activity of these genes can be demonstrated by electrophoretic mobility shift assay or yeast one-hybrid system. Once the binding specificity is confirmed, the target genes containing the specific regulatory sequences can be identified. Constitutive expression and/or antisense inhibition of these genes in *Poncirus* may reveal the role of these genes in cold hardiness In addition, since these genes were not induced or induced little in response to cold in pummelo, overexpression of these genes in cold-sensitive *Citrus* species may improve their cold tolerance

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BIOGRAPHICAL SKETCH

Mehtap Şahin-Çevik was born in Malatya, Turkey in 1972. Ten years later she moved to Ankara and completed her high school education there. She graduated from the College of Agriculture at Ankara University with B. S. degree in horticulture in June 1994. One year after graduation, in July 1995, she was awarded a scholarship by the Turkish Ministry of Education to pursue M.S. and Ph.D. degrees in the field of Horticulture in the USA. Before coming to the USA, she studied English for 6 months at Middle East Technical University in Ankara, Turkey. In May 1996 she came to Gainesville, Florida, and attended the English Language Institute at the University of Florida for 1 year. She began her master's degree program in the Horticultural Sciences Department in May 1997 and worked on molecular markers and QTL mapping in Citrus. After finishing her master's degree program, she started her Ph.D. program in the Horticultural Sciences Department in May 1999. She is expected to graduate in December 2003.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy

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